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Characterisation of CTX-M-β-Lactamases in Enterobacteriaceaeae in hospitals in Kuwait

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Abstract

Introduction: In this decade, the CTX-M family of enzymes are considered to be the most common type of extended-spectrum β-lactamases (ESBLs). The production of these Class A β-lactamases are noted to be most prevalent in the *Enterobacteriaceaeae* family. Many global reports indicated that CTX-M-15, of the CTX-M-1 group, is a growing concern, causing resistance to different classes of antibiotics. Worrisome trends of the spread of this enzyme have been indicated in nosocomial and community settings worldwide. Moreover, the same predicament is faced along the Middle East area, especially in the absence of restricted antibiotic usage policies. Many reports from Kuwait indicated the spread of a multi-drug resistant (MDR) $bla_{\text{CTX-M-15}}$ gene in different hospitals. $bla_{\text{CTX-M-15}}$ genes are often known to be carried on large transferable plasmids. Usually, the mobilization of these plasmid-encoded enzymes is carried out by insertion sequence like IS*Ecp1*.

Aims: This work aims to investigate the distribution of $bla_{\text{CTX-M}}$ genes in five major hospitals in Kuwait and to study and analyse the genetic environment of the described $bla_{\text{CTX-M}}$ genes.

Materials and methods: One hundred and seven isolates of *E. coli* (84) (78.50%) and *K. pneumoniae* (23) (21.49%) were collected between 2006 and 2010 from five distantly located hospitals in Kuwait. All of the collected isolates were identified as ESBL-producers using Vitek 2 system. The production of cefotaximases was detected using disc diffusion with cefotaxime and clavulanic acid according to Clinical and Laboratory Standards Institute (CLSI) criteria. Conformation of CTX-M

production was maintained by PCR amplification and further sequencing. The minimum inhibitory concentrations (MICs) of the collected isolates were determined by the double dilutions agar method described by the CLSI. Four different classes of antibiotics were used (aminoglycosides, different generations of cephalosporins, fluoroquinolones, and 3 different carbapenems). The genotypic relatedness of the collected strains was assessed by the use of an enhanced Pulsed-field gel electrophoresis (PFGE) method. Further amplifications with primer walking and simplex PCR were done to seek the genetic context of the MDR strains. S1 nuclease was used to size plasmids carrying the described $bla_{\text{CTX-M}}$ genes and conjugation studies were implemented to detect the transferability of the plasmids carrying the reported $bla_{\text{CTX-M}}$ genes.

Results: All of the collected strains showed to be ESBL-producers and in particular cefotaximases-producers. Upon amplification, CTX-M-1 group was the only CTX-M-group present in the collected strains. When sequenced, $bla_{\text{CTX-M-15}}$ was found to be the most prevalent. In addition, strains carrying the $bla_{\text{CTX-M-3}}$ gene were identified, these have previously been found in the Middle East; however, this thesis has the first descriptions of $bla_{\text{CTX-M-28}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-117}}$ in this region. After the determination of the MICs of the collected strains, 94 (87.85%) were resistant to cefepime, 107 (100%) to cefotaxime, 48 (44.85%) to cefoxitin, 78 (72.89%) ciprofloxacin, and 71 (66.35%) to gentamicin. All of the strains were susceptible to carbapenems. Twenty-eight strains (26.2%) showed MDR pattern. With the enhanced PFGE method, only 22 isolates exhibited banding patterns that allowed grouping them into 10 distinct PFGE clusters. Notably, strains sharing ≥85% were

from the same hospitals (isolates 1 with 2, 21 with 22, and 91 with 92 from the maternity hospital (M), 52 with 53 from Kuwait Oil Company hospital (KOC), 78 with 79 and 83 with 84 from Infectious Diseases Hospital (IDH), 97 with 98 and 95 with 96 from Al-Amiri hospital(A)). Primer walking and simplex PCR experiments used for the genetic environment studies yielded 7 different genetic constructions for the described bla_{CTX-M} genes. All of the described bla_{CTX-M} genes were carried on plasmids ranging in size from 60 – 271 kb. Only 3 of the selected strains were of IncFII and the rest wereindeterminate. Possibly, two $bla_{CTX-M-15}$ genes are likely to be carried on the chromosome. All of the described bla_{CTX-M} genes are considered to be transferable except for $bla_{CTX-M-28}$. The sizes of the conjugative plasmids and incompatibility groups are the same as their parental plasmids.

Conclusion: In conclusion, $bla_{CTX-M-15}$ is the most common ESBL gene found in Kuwaiti hospitals. It is also causing a MDR pattern with resistance to 3 different generations of cephalosporins and to two other classes (aminoglycosides and gentamicin), but sensitive to carbapenems. This led to restricting the treatment option into carbapenems. Antibiotic selective pressure could have played a major role in the development of $bla_{CTX-M-15}$ derivatives such as $bla_{CTX-M-28}$, $bla_{CTX-M-55}$, and $bla_{CTX-M-117}$. The probable explanation of the spread of $bla_{CTX-M-15}$ is horizontal gene transfer carried by IS*Ecp1* and the conjugative properties of the plasmids carrying $bla_{CTX-M-15}$. Variability of the genetic environments obtained explains the non-conditional existence of IS*Ecp1* to the ''W'' region. Absence of the IS*Ecp1* in one of the reported structures of $bla_{CTX-M-15}$ genetic contexts is noted. Therefore, the

existence of *bla*$_{\text{CTX-M-15}}$ could be due to the presence of another insertion sequences downstream or as a part of a larger gene cassette.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

No portion of the work referred to in this thesis has been submitted in support of an application for another degree.

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Papers presented

Characterization of IncFIA, IncFIB, and IncN Plasmids Carrying CTX-M-3, -15, -55 β-lactamases from *Escherichia coli* and *Klebsiella pneumoniae* Strains from 5 Major Kuwaiti Hospitals

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Abbreviations

A	Amiri
Å	Angstroms
A. baumannii	*Acinetobacteri baumannii*
AR	Antibiotic resistance
B	beta
Bla	beta-lactamase
BLAST	Basic Local Alignment Search Tool
BSAC	British Society of Antimicrobial Agents and Chemotherapy
CLS	Cell lysis solution
CLSI	Clinical Laboratory Standards Institute
CMA	Certified megabase agarose
CTX-M	Cefotaxime
DNA	Deoxyribonucleic acid
E. aerogenes	*Enterobacter aerogenes*
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EMBL	European bioinformatics institute
ESBL	Extended-spectrum-β-lactamase
EUCAST	European Committee on antimicrobial susceptibility testing
EXPASY	Expert protein analysis system
G	gram
GW-PCR	Genome walking-polymerase chain reaction
HPA	Health Protection Agency
HGT	Horizontal gene transfer
IB	Ibn-sina

ID	Infectious diseases
Inc	Incompatibility
K.	*Kluyvera*
Kb	Kilo-base pairs
KOC	Kuwait oil company
K. pneumoniae	*Klebsiella pneumoniae*
L	Litre
MBLs	Metallo-β-lactamases
MGE	Mobile Genetic elements
MDR	Multi-drug resistance/resistant
Mg	Milligram
MIC	Minimum inhibitory concentration
mL	Milli-Litre
mM	Milli-Molar
M	Molar
MGE	Mobile genetic elements
µ	micro
µg	microgram
NCBI	National Centre of Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
P. aeruginosa	*Pseudomonas aeruginosa*
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Measure of acidity or basicity of a solution
PK	Proteinase K
pmole	Pico-moles

Rpm	Revolutions per minute
S	seconds
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulphate
Spp.	species
TAE	Tris/acetic acid/ethylenediaminetetraacetic acid
TBE	Tris/Borate/ethylenediaminetetraacetic acid
TE	Tris/EDTA
U	Units
UTI	Urinary tract infection
V	Volts
w/v	Weight by volume

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1 Introduction

1.1 Prologue

During the Second World War, antimicrobial drugs were introduced as sulfonamides and penicillins. The rise of this antimicrobial era developed into using newer β-lactams. It was also considered to be the ‘’golden age’’ of antimicrobials (Biondi *et al.*, 2011). After 40 years of extensive use of antibiotics, major problems with β-lactam drugs were reported (Andersson *et al.*, 2001, Biondi *et al.*, 2011). It was formidable how bacterial resistance developed with proficiency to become a serious concern and a source of threat in the medical settings (Andersson *et al.*, 2001, Canton *et al.*, 2012, Chong *et al.*, 2011, Walsh and Wright, 2009). Bacterial resistance to antimicrobial drugs was discerned to be a worldwide phenomenon that led to dramatic increase in morbidity, mortality, and treatment costs; affecting the medical field, public health, and economy (Frost *et al.*, 2005, Hawkey and Jones, 2009). The study of bacterial resistance to antimicrobial drugs must be increased (Novais *et al.*, 2010).

1.2 Antibiotic Resistance: *a closer look*

1.2.1 Consensus definition of resistance

The term ‘’susceptibility’’ can be construed by two different means; biological or pharmacological (Livermore, 1995). The biological term defines a bacterial pathogen as resistant if the minimum inhibitory concentrations (MICs) exceed the reference values regardless of the resistance mechanism. On the other hand, the pharmacological definition of susceptibility defines a bacterial pathogen as resistant in relation to the drug concentration achieved *in vivo* and considers the resistance mechanism. Large clinical organization bodies such as the Clinical Laboratory Standards Institute (CLSI),the British Society for Antimicrobial Chemotherapy (BSAC) (Livermore, 1995), and European committee on antimicrobial susceptibility testing (EUCAST) use the pharmacological definition of susceptibility rather than the biological term. The CLSI (Previously called the National Committee for Clinical Laboratory Standards or NCCLS), in the United States, publishes the guidelines for setting the breakpoints required for the definition of susceptibility. On the other hand, BSAC publishes those breakpoints in the United Kingdom and EUCAST in the European countries. The above mentioned bodies set the breakpoints required to define a pathogen as (susceptible, intermediate, or resistant) based on the knowledge of assessing the pharmacokinetics and pharmacodynamics of the antibacterial used, the wild-type distribution of MICs, and the clinical outcomes of infections when the antibacterial is used (Turnidge and Paterson, 2007). The biological definition of susceptibility is used by clinicians and microbiologists.

A noteworthy fact is that there are no single standard criteria for the definition of breakpoints, different countries define breakpoints with different criteria (Barger *et al.*, 2003). The breakpoint values set by the CLSI are usually higher that those set by BSAC (MacGowan and Wise, 2001). The differences between the values of the breakpoints set by BSAC, CLSI, or EUCAST mainly due to the differences in the guidelines used for therapy. These differences are also due to different resistant rates reported by different surveillance studies even when the MIC distribution is the same.

The standards used for defining the breakpoints are usually (susceptible, intermediate, and resistant). Generally, susceptible is implied to a bacterial pathogen that will probably respond to antibiotics. The term ‘‘intermediate’’ is commonly used to a bacterial pathogen that is less likely to respond to antibiotics and, in some cases, would responds to increased doses. A bacterial pathogen is defined as resistant when it does not respond to antibiotics (MacGowan and Wise, 2001). The guidelines are established by recognised standard-setting organizations (such as the CLSI) and are based on the analysis of achievable serum levels, in vitro susceptibilities of bacteria, and human clinical trials (Rice, 2012).

Despite the use of the pharmacological definition of susceptibility by recognised bodies (such as the BSAC), the biological definition is preferred to be used on the clinical basis. The use of the biological definition can be justified by the fact that this definition signifies the reduction of the MICs even if it is small and enables clinicians to monitor such incidents. On the other hand, the pharmacological definition could

disregard such change, leading to mistakenly considering a bacterial pathogen susceptible or intermediate when it is gaining resistance. Therefore, affecting the therapeutic choice making (Livermore, 1995).

The term ''bacterial resistance'' used in both definitions is not only governed by the MIC, but also by other factors. These include the drug concentration at the site of infection and the metabolic state of the infection encountered. A simple example of the latter is when an organism is susceptible *in vitro* but, if present in biofilms attached to a foreign body, it behaves as resistant (Rice, 2012). In other words, there is no firm definition of resistance that can be applicable to all bacterial pathogens. Instead, to make appropriate decision for treatment, the clinical cases and the variability of the individual clinical circumstances should all be taken into account (Rice, 2012).

1.2.2 The spread of antibiotic resistance

The onset of antimicrobial resistance has limited appropriate choice making in antibiotics used for treatment (Bush, 2010, Lee *et al.*, 2012). Moreover, these infections are not only restricted to hospital settings; rather, they infiltrate into the community as well (Bush, 2010, Rice, 2012). In fact, the trend in which antibiotic resistance is developing is worrisome and is precluding the use of most antimicrobial agents available in the market (Andersson *et al.*, 2001, Rice, 2012). Furthermore, it has been officially stated that the spread of antibiotic resistant bacteria represents a ''growing challenge'' for the development of newer

antimicrobial agents capable of concurring this problematic issue (Bassetti *et al.*, 2011b, Chroma and Kolar, 2010).

1.2.3 Genetics of antibiotic resistance

The development of antibiotic resistance is considered to be an evolutionary response made by bacteria naturally, upon the exposure to antimicrobial drugs. In fact, it is a bacterial populations's means of survival in the face of the inhibition concentration of antimicrobials. Bacterial resistance may be either inherited, or acquired by various mechanisms (Chroma and Kolar, 2010, Livermore, 2003). Intrinsic bacterial resistance is most commonly involved with chromosomal resistance mechanisms, the presence of efflux pumps, or the lack of affinity for the target drug and is unique to a certain bacterial genus (Chroma and Kolar, 2010, Giedraitiene *et al.*, 2011). An example of intrinsic (innate) bacterial resistance is the resistance of *Escherichia coli* (*E. coli*) to vancomycin (Giedraitiene *et al.*, 2011). On the other hand, bacteria from different species may acquire resistance by (i) exogenous DNA transfer, (ii) mutation of cellular genes, or (iii) a combination of both mechanisms (Giedraitiene *et al.*, 2011, Livermore, 2003). The acquisition of exogenous resistance determinants applies to transferable plasmids, chromosomes, transposons, integrons, and bacteriophages (Giedraitiene *et al.*, 2011, Rice, 2012). Antimicrobial resistance genes are often plasmid-mediated. The main mechanism by which resistance genes are transferred from one bacterium to another is by the transfer of plasmids (Giedraitiene *et al.*, 2011). This mode of transfer is called

''horizontal gene transfer'' (HGT). Moreover, HGT is thought to be the main causative agent for the transmission and dissemination of multi-drug resistant bacterial pathogens. It is remarkable how horizontal transfer influence bacterial communities and allows the spread of different resistance patterns as well as epidemics (Bassetti *et al.*, 2011b). Nevertheless, horizontal transfer plays a major role in bacterial diversity and evolution (Livermore, 2003, Slater *et al.*, 2008). However, the factors that favour this mode of transfer are poorly understood (Livermore, 2003).

1.3 β-lactam drugs

One of the most widely used antibiotics is β-lactam antibiotics. Penicillin was the first β-lactam drug discovered (reviewed by Andersson, 2001). Later, newer β-lactams have been discovered and used to treat infections with Gram-positive and Gram-negative bacteria (Biondi *et al.*, 2011). β-lactam antimicrobials are not active against species that lack cell wall in their structure (such as *Chlamydia* spp) or bacterial strains with impermeable cell walls (such as *Mycobacterium* spp.). In addition, β-lactam drugs are also not active against intracellular bacterial species (such as *Brucella* or *Legionella*). However, these antimicrobials share high therapeutic index and are thought to be safe to be used even in pregnancy (Mims, 2004). Most likely, the usage of these drugs is for the treatment of different infections as well as urinary tract infections as they are eliminated unchanged by the kidneys (Page, 2002).

β-lactam drugs are antimicrobial agents possessing a four-membered nitrogen-containing β-lactam rings, usually hydrolysed by β-lactamases (Samaha-Kfoury and Araj, 2003). The main action of these antimicrobial agents is targeted on the bacterial cell wall synthesis (Page, 2002). In fact, the β-lactam ring inhibits the cell wall synthesis by binding to cell wall synthesis enzymes known as penicillin-binding-proteins or transpeptidases (PBPs) (Page, 2002, Rice, 2012). PBPs are thought to be responsible for the action of cross-linkage of the structure of the bacterial cell wall. The binding of β-lactams to PBPs inhibit their action resulting in the accumulation of the cell units and inhibition of cell growth. Eventually, leading to the activation of the cell's autolysis system and ultimate cell lysis (Mims, 2004, Rice, 2012). The action of β-lactams is considered to be bactericidal. β-lactam drugs consist of four major groups that differ from one another by the presence of additional rings. These groups are; penicillins, cephalosporins, carbapenems, and monobactams. The additional ring present in each group is; thiazolidine ring in penicillins, cephem nucleus in cephalosporins and a double ring structure in carbapenems. By contrast, monobactams do not contain an additional ring to the β-lactam ring (Samaha-Kfoury and Araj, 2003). The basic chemical structures of the mentioned β-lactams are seen in Figure 1. Each group of β-lactam drugs consist of sub-groups. Penicillins consist of Penicillin G, penicillin resistant penicillins, aminopenicillins, carboxypenicillins, or ureidopencillins. Moreover, there are four generations of cephalosporins (reviewed by Harrison 2008). In case of carbapenems, they consist of imipenem, meropenem, and ertapenem. The only monobactam is aztreonam

(Samaha-Kfoury and Araj, 2003). The β-lactam group and their examples are simplified in Table 1 (Mims, 2004, Samaha-Kfoury and Araj, 2003).

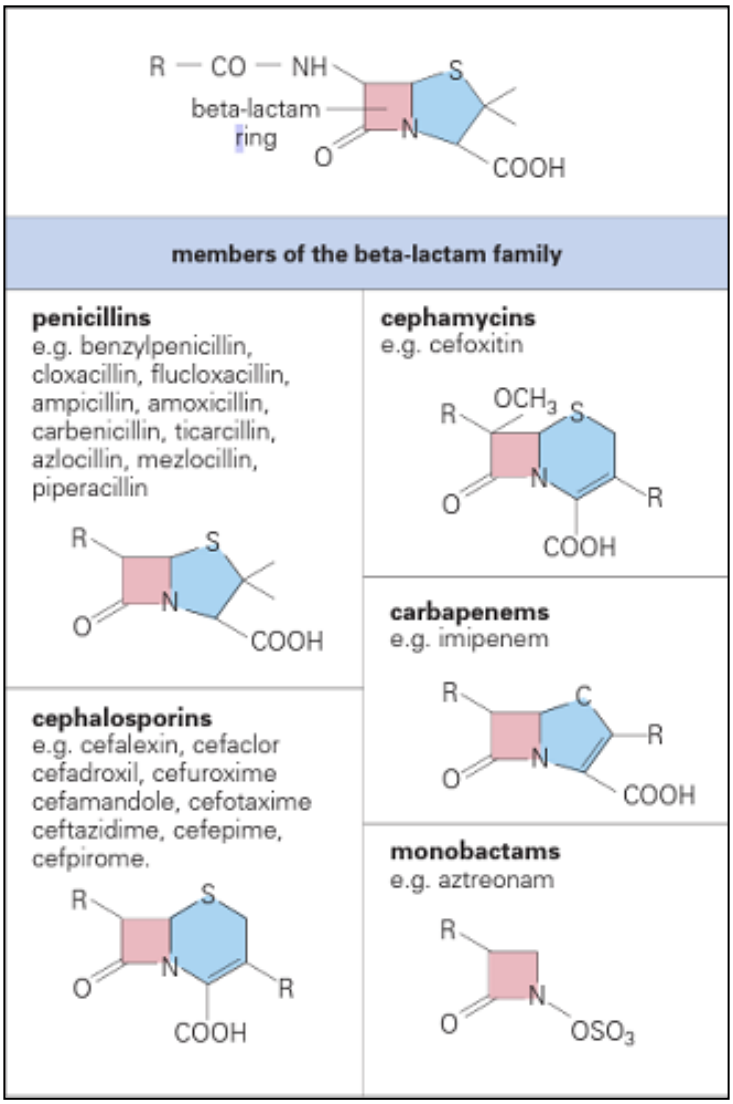


Figure 1:The basic chemical structure of β-lactam family. The common part is the β-lactam ring in all β-lactam family. The R denotes the sites of variation of the additional ring that is different in each group. Penicillins and cephalosporins represent major groups of β-lactams. This structure is adapted from Mims (2004).

B-lactam drug	Category	General spectrum of activity	Examples of the drug
Penicillins	Natural	Gram-positive bacteria	Penicillin G, V
	Semisynthetic (β-lactamase resistant) penicillin	Gram-positive bacteria (including β-lactamase producers)	Cloxacillin, dicoxacillin, nafcillin, and oxacillin
	Semisynthetic (amino) penicillin	Gram-positive bacteria Gram-negative bacteria (including spirochetes, *Proteus mirabilis*, and some *E. coli*)	Amoxacillin and ampicillin
	Semisynthetic (carboxy) penicillin	Gram-positive bacteria with enhanced coverage of Gram-negatives, including *Pseudomonas* and *Klebsiella*	Carbenicillin, ticarcillin, mezlocillin, and piperacillin
	Semisynthetic (uredio) penicillin		
Cephalosporins	First generation	Gram-positive bacteria	Cefadroxil, cefazolin, cephalexin, cephalothin, and cephradine.
	Second generation	Gram-positive bacteria	Cefaclor, cefamandole, cemetazole, cefonicid, cefotetan, cefprozil, cefuroxime, and cephamycin*
	Third generation	Gram-positive bacteria	Cefdinir, cefditoren, cefoperazone, cepodxime, cefotaxime, ceftazidime, ceftibuten, ceftizoxime, and ceftriaxone
	Fourth generation	Improved activity against Gram-negative bacteria	Cefepime, cefpirome

B-lactam drug	Category	General spectrum of activity	Examples of the drug
Carbapenems		Gram-negative bacteria especially ESBL-producers	Ertapenem, imipenem, and meropenem
Monobactams			Aztreonam

Table 1: β-lactam groups and most commonly used types. *cephamycin can be considered as a group of cephalosporins or as a second generation cephalosporin. Cefoxitin, cefotetan, and cefmetazole are examples of cephamycins

1.4 β-lactam resistance

β-lactam resistance is caused by four main mechanisms; the production of β-lactamases that bind and hydrolyse β-lactam drugs, alteration of the active site of PBPs, decreased expression of outer membrane proteins (OMPs) that leads to reduced permeability, reducing access to PBPs, and an induction of an efflux pump leading to the export of relevant substrates from the periplasm of the bacteria to the surrounding area (Drawz and Bonomo, 2010, Page, 2002, Rice, 2012, Samaha-Kfoury and Araj, 2003). Another mechanism of resistance to β-lactam drugs is the alteration of the target sites PBPs. This mechanism is mostly encountered when dealing with methicillin-resistant bacteria (Chambers, 2001, Llarrull *et al.*, 2010). The third and fourth mechanisms are most commonly encountered in Gram-negative bacteria (Llarrull *et al.*, 2010, Page, 2002).

The production of β-lactamases is thought to be the major threat to β-lactam antibiotics (Andersson *et al.*, 2001, Bassetti *et al.*, 2011b, Biondi *et al.*, 2011, Drawz and Bonomo, 2010, Oteo *et al.*, 2010). Inevitably, the alarming trends of the presence of β-lactamases are becoming the challenge of the antibiotic era. Moreover, β-lactamase enzymes such as bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ are reported to be the main causative agents for β-lactam drugs resistance (Bassetti *et al.*, 2011b, Paterson and Bonomo, 2005). Interestingly, the spread of these enzymes by transferable genetic elements in *Enterobacteriaceaeae* is magnifying this challenge faced in the infectious disease communities (Bassetti *et al.*, 2011b, Bush, 2010, Malloy and Campos, 2011). Not only have the infectious disease communities addressed this issue, but also the Infectious Diseases Society of America identified Enterobacteriaceaeae as one of the most β-lactam resistant pathogens being difficult to treat (Lee *et al.*, 2012).

1.5 Extended spectrum β-lactamases (ESBLs): *a brief history*

Bacterial strains can adapt different mechanisms for mediating β-lactamase resistance (Drawz and Bonomo, 2010, Page, 2002, Rice, 2012, Samaha-Kfoury and Araj, 2003). ESBLs are considered to be only a subset of β-lactamases but not representing all (Malloy and Campos, 2011). The emergence of the first clinical β-lactamase enzyme ‘’TEM-1’’ was documented in the early 1960s in association with the use of ampicillin (reviewed by Medierios, 1984). Although the actual first β-lactamase was identified earlier in 1940 before the introduction of penicillin

(Abraham and Chain, 1940). TEM-1 enzyme was isolated from a patient in Greece named Temoneira, therefore the designation ''TEM'' was used (Medeiros, 1984). It was believed that TEM-1 was plasmid-mediated β-lactamase enzyme. More importantly, the spectrum of activity of TEM-1 enzyme towards β-lactam drugs was noted to be narrow and only resistant to ampicillin (Datta and Kontomichalou, 1965). Within a few years of the TEM-1 report and in 1972, the first description of bla_{SHV-1} was documented (reviewed by Du Bois *et al*, 1995) (Du Bois *et al.*, 1995). The designation of SHV is for the presence of sulphydryl variable group within this family of enzymes (Pitton, 1972). In the 1980's, different reports of ESBL-enzymes of TEM and SHV-types with extended-spectrum-activity towards cephalosporins were described (Jacoby and Medeiros, 1991, Rice, 2012). The spread of CTX-M family of β-lactamases started in 1990 (Bauernfeind *et al.*, 1990, Tzouvelekis *et al.*, 2000) and increased ever since becoming the most prevalent family of β-lactamases described in *E. coli* (Qi *et al.*, 2010). Shortly, after the accumulated reports of CTX-M enzymes, carbapenemases were described (Gupta *et al.*, 2011, Queenan and Bush, 2007) (Figure 2).

1.6 ESBLs definition

The term ''ESBLs'' was referred to later β-lactamases that were discovered after the emergence of SHV-1 and were closely related to TEM-1 and TEM-2 enzymes but conferred resistance to broadened-spectrum cephalosporins (Paterson and

Bonomo, 2005). Most commonly, plasmid-mediated ESBLs of the TEM, SHV, and CTX-Ms are described (Boyd *et al.*, 2004). Debates around a certain definition of

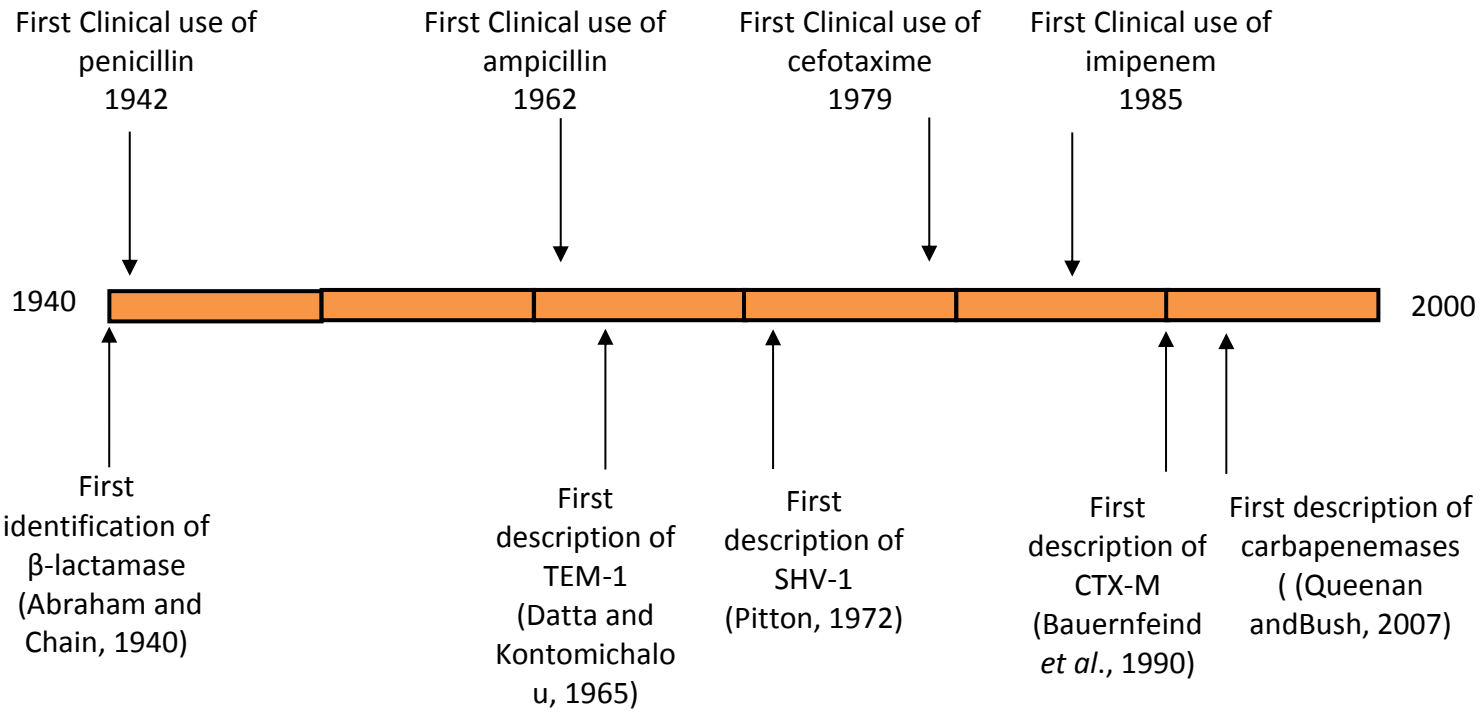


Figure 2: Time line figure showing the first description of the first member of β-lactamase family with the first introduction of the β-lactam drug. This figure was adapted from (Rice, 2012).

ESBLs have arisen, and different β-lactamase investigators proposed different definitions (Ambler *et al.*, 1991, Bush *et al.*, 1995, Bush *et al.*, 2009, Bush and Jacoby, 2010, Giske *et al.*, 2009, Lee *et al.*, 2012). Regardless, the Health Protection Agency (HPA) did define ESBLs but that definition did not cover all ESBLs. The HPA stated that infections-producing ESBLs are resistant to cephalosporins, penicillins, fluoroquinolones, trimethoprim, tetracycline and some other antibiotics limiting treamtment options to nitrofurantoin and fosfomycin http://www.hpa.org.uk last accessed (15th August 2012).

Yet, scientists could not depend on the definition of ESBLs set by the HPA. The issue of scientists not being able to maintain a specific definition of these enzymes is due to two main reasons; one of which is the wide substrate specificity of these enzymes' active site, and the other is that ESBLs are constantly developing with rapid, complex, and dynamic evolution (Gniadkowski, 2001). Therefore, the resistance pattern of ESBLs is constantly changing over the years, leading to the use of a definition that is probably subject to change whenever their resistance profile changes (Paterson and Bonomo, 2005). Nevertheless, ESBLs can be defined as a group of acquired (transferable) rather than inherited β-lactamases capable of hydrolysing a broad spectrum of oxyimino-β-lactam drugs such as penicillins, expanded-spectrum of cephalosporins, and monobactams but not cephamysins or carbapenems. Mainly their action is inhibited by the presence of a site directed-β-

lactamase inhibitor such as clavulanic acid (Bush *et al.*, 2009, Bush and Fisher, 2011, Gniadkowski, 2001, Livermore, 2008, Paterson and Bonomo, 2005).

1.7 ESBL classification

Ever since the discovery of the first β-lactamase enzyme ''TEM-1'', increasing number of reports of ESBLs of different types have been documented in literature (Gniadkowski, 2001, Samaha-Kfoury and Araj, 2003). At present, more than 1200 different ESBLs variants are known (http://www.lahey.org/studies/webt.htm) last accessed (15th August 2012). These ESBL variants have been classified into fifteen distinct structural/evolutionary families on the basis of similarities between their amino acid sequences. These families are OXA, TEM, SHV, CTX-M, CMY, AmpC, imp, VIM, GES, Carb, KPC, IND, PER, VEB, and SME (Gniadkowski, 2001) (http://www.lahey.org/studies/webt.htm) last accessed (15th August 2012).

Three classification schemes are used to classify β-lactamases; Ambler molecular classification scheme (Ambler *et al.*, 1991), a functional classification scheme adopted by Bush-Jacoby (Bush *et al.*, 1995, Bush and Jacoby, 2010), and a clinical classification proposed by Giske (Giske *et al.*, 2009). Different studies used to classify β-lactamase genes (*bla*) on the basis of their genetic location. However, this classical classification system is no longer of use. Owing to the fact that plasmid-

mediated *bla* genes can be mobilized and integrated into the chromosome. Another reason is the exclusive localization of the *bla* genes on the chromosome or the plasmid solely without being mobilized and integrated to one another (Canton *et al.*, 2012, Coelho *et al.*, 2010, Toleman *et al.*, 2006). β-lactamase genes are mainly plasmid-mediated. But also, in Gram-negative bacteria, *bla* genes are commonly found on the chromosome (Canton *et al.*, 2012).

Each classification system has limitations especially in the face of new resistance patterns and the discovery of new ESBL families (Giske *et al.*, 2009). Therefore, classification systems have to be updated as the definition of the ESBL enzymes is evolving in relation to their resistance pattern (Hall and Barlow, 2005).

1.7.1 The Ambler molecular classification system

The Ambler classification scheme categorizes ESBLs on the basis of amino acid sequence similarities (Bush and Fisher, 2011). It is also described to classify ESBLs according to the enzymes' active site (Malloy and Campos, 2011). Ambler classification is the most commonly used scheme especially by scientists, but it renders confusion to clinicians and microbiologists (Gniadkowski, 2001, Hall and Barlow, 2005, Hall and Barlow, 2004, Singh *et al.*, 2009). This issue was faced due to the diversity of the molecular and functional characteristics of ESBLs (Singh *et al.*,

2009). ESBLs may inherit the molecular active site structure from their parent enzymes but with wider substrate-binding cavity leading to the increase in the range of their hydrolytic activity (Gniadkowski, 2001). The classical Ambler scheme focuses on the structure of the enzyme and neglects its function Therefore, some ESBLs with certain amino acid substitution can be mis-classified into a certain Ambler group having different functionality (Bush and Fisher, 2011).

Ambler classification categorizes ESBLs into four groups; A, B, C, and D (Ambler *et al.*, 1991, Hall and Barlow, 2005). Originally, Ambler specified only two classes; class A, where the active site is serine β-lactamases, and class B, which are metallo-β-lactamases (MBLs) requiring a bivalent metal ion (usually Zn^{+2}) for their action (Ambler *et al.*, 1991). An updated Ambler classification uses four classes, class A, C, and D with serine acting as the active site and sharing little sequence similarities (Ambler *et al.*, 1991). The three Ambler classes A, C, and D share high structural similarities (Ambler *et al.*, 1991, Hall and Barlow, 2005, Hall and Barlow, 2004). Ambler class A-β-lactamases are known to be penicillinases, while class C β-lactamases are known to be AmpC-β-lactamases and both with serine serving as the active site (Jaurin and Grundstrom, 1981) with cephalosporin as the preferential substrate. Another class with Ambler classification that has serine acting as the active site is class D, these β-lactamases are OXA-ESBLs (Ouellette *et al.*, 1987). All of the Ambler classes with serine active site have different origins (Hall and Barlow, 2005). This made them to be grouped into different classes despite the same active

site (Hall and Barlow, 2005). Previously, Ambler classification considered all class B (MBLs) are the same and closely related. However, this problem was addressed and corrected in the updated Ambler classification. The revised Ambler classification proposed that class B-ESBLs are divided into sub-groups (i.e. B1, B2, and B3) based on their sequence similarities (Hall and Barlow, 2005). The sub-groups serve those ESBLs that hydrolyse carbapenems escaping the serine active site (MBLs). The discovery of new MBLs sharing less amino-acid sequence similarity with earlier members led to the need to update the Ambler classification system (Hall and Barlow, 2005, Singh *et al.*, 2009). The updated Ambler classification scheme is presented in Figure 3.

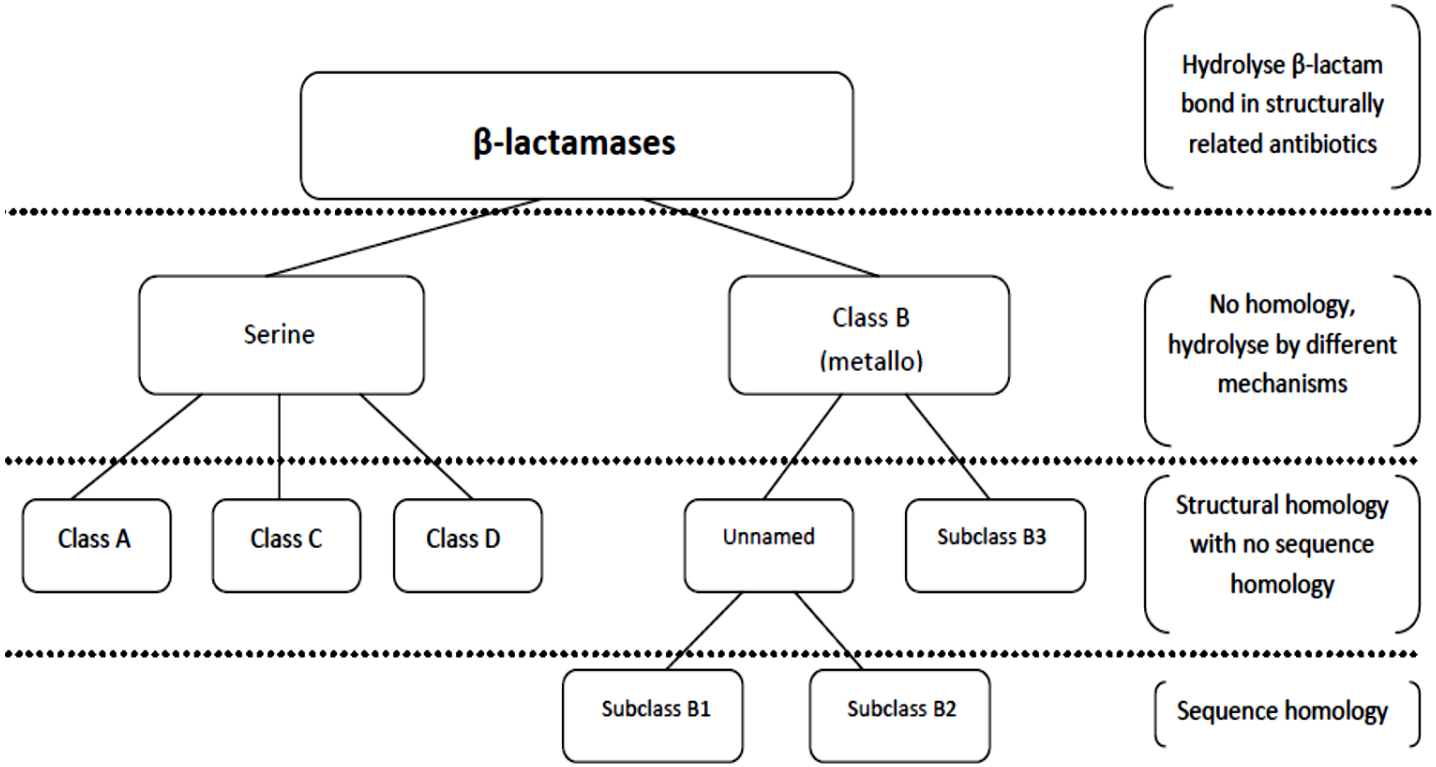


Figure 3: Schematic diagram of the updated Ambler classification system.

1.7.1 The Bush functional classification

The functional classification of ESBLs was originally proposed in 1973 (Richmond and Sykes, 1973). Later in 1995, Bush maintained to classify ESBLs based on their hydrolytic and inhibitory profiles (Bush *et al.*, 1995). Nevertheless, not all β-lactamases (in certain species) were successfully classified with the Bush classification. Some showed to be indistinguishable with the functional classification (Gniadkowski, 2001). Also, upon the discovery of newer ESBLs with expanded and variable hydrolytic and inhibitory profiles, there was a need to update the functional classification (Bush and Jacoby, 2010, Malloy and Campos, 2011). Hence, the Bush classification was updated in 2010 (Bush and Jacoby, 2010). Admittedly, grouping ESBLs based on their functionality is more subjective than classifying them based on their protein structure (as in Ambler classification). The functional classification provides clinicians and microbiologists a more practical mean to correlate the properties of a specific ESBL member with the results of the susceptibility testing of a clinical isolate. In the Bush classification, ESBLs are classified into 3 groups; group 1, group 2, and group 3. Group 1 represents cephalosporinases not inhibited by clavulanic acid, which belongs to Ambler class C. Group 1 has a subgroup (i.e. 1e) which has an expanded-spectrum of activity to cephalosporins. Group 2 serine-β-lactamases includes Ambler class A and D and represents most of the ESBLs. The functional group 2 preferentially hydrolyse benzylpenicillin and many penicillin derivatives and not necessarily inhibited by clavulanic acid. It is sub-divided into eleven sub-groups (2a, 2b, 2be, 2br, 2ber, 2c, 2d, 2de, 2df, 2e, and 2f). The subdivision of each group was maintained on the basis

of the hydrolytic activity towards antibiotics and inhibition by β-lactam inhibitors, indicating variable activity of the members of the sub-divisions towards extended-spectrum β-lactam drugs and β-lactam inhibitors (Bush and Jacoby, 2010). The most widely spread β-lactamases (such as $bla_{\text{CTX-M-15}}$) fall into functional group 2be. This functional group shows increased hydrolytic activity towards extended-spectrum oxyimino-β-lactams (such as cefotaxime, ceftazidime, ceftriaxone, cefepime, and aztreonam). Also, they are distinguished by their susceptibility towards clavulanic acid and tazobactam, but resistance towards ethylenediaminetetraacetic acid (EDTA) (Bush and Jacoby, 2010). Functional group 3 is mainly composed of metallo-β-lactamases (MBL) (Bush and Jacoby, 2010). The updated Bush functional scheme is aligned with the molecular Ambler scheme with few examples of the most commonly encountered ESBLs enzymes representing each group (Table 2).

Bush-Jacoby group	Molecular class	Selected enzymes (most common)
1	C	*E. coli* and *Pseudomonas aeruginosa* (*P. aeruginosa)* AMPC, CMY-2, FOX-1, MIR-1, P99
1e	C	GC1, CMY-37
2a	A	PC1 and other staphylococcal penicillinase
2b	A	SHV-1, TEM-1, TEM-2, TLE-1 (TEM-90)
2be	A	CTX-M-15, CTX-M-44 (Toho-1), PER-1, SFO-1, SHV-5, TEM-10, TEM-26, VEB-1
2br	A	TEM-30, TEM-76, TEM-103, SHV-10, SHV-26
2ber	A	TEM-50, TEM-68, TEM-89
2c	A	PSE-1, CARB-3
2d	D	OXA-1, OXA-10
2de	D	OXA-11, OXA-15
2df	D	OXA-23, OXA-48
2e	A	CapA
2f	A	IMI-1, KPC-2, KPC-3, SME-1, GES-2
3a	B	IMP-1, L1, NDM-1, VIM-1
3b	B	CphA, Sfh-1

Table 2: Updated functional classification of ESBLs aligned with Ambler classification.

1.7.2 Clinical classification of ESBLs

The establishment of the clinical classification of ESBLs was to simplify the definition of these enzymes to clinicians and to the infection control use (Giske *et al.*, 2009). For scientific purposes, the designation of the enzyme with the enzyme's family would be sufficient (Livermore, 2008). The proposal of the clinical scheme for classifying ESBLs was not to replace the Ambler or the Bush classification. It was set to complement the two, mainly for the use of health care professionals. Therefore, many differences within each group established by this classification may be neglected. Moreover, operational criteria are approached in this mode of categorization. These approaches are mainly of clinical use, such as the detection of the group of enzymes and the synergy with clavulanate. In the clinical classification ESBLs are categorized into three main classes; $ESBL_A$, $ESBL_M$, and $ESBL_{CARBA}$. $ESBL_A$ are mainly those ESBLs not susceptible to expanded-spectrum cephalosporins and show synergy with clavulanate. On the other hand, $ESBL_M$ are those ESBLs which represent miscellaneous group. The latter group is subdivided into $ESBL_{M-C}$ representing plasmid-mediated AmpC class C. Another sub-division of $ESBL_M$ is $ESBL_{M-D}$, which are mainly OXA-ESBLs of class D. The third category of the clinical classification is $ESBL_{CARBA}$ which members show activity against carbapenems (Giske *et al.*, 2009). The clinical classification of ESBLs can be seen in Table 3.

	$ESBL_A$	$ESBL_M$	$ESBL_{CARBA}$
β-lactamase class	High prevalent $ESBL_A$ CTX-M TEM-ESBLs SHV-ESBLs VEB PER	$ESBL_{M-C}$ (plasmid-mediated AmpC) $ESBL_{M-D}$ (OXA enzymes)	$ESBL_{CARBA-A}$ $ESBL_{CARBA-B}$ (MBL) $ESBL_{CARBA-D}$ (OXA)
Definition	Non-susceptible to extended-spectrum cephalosporins Show synergy with clavulanate	Non-susceptible to extended-spectrum cephalosporins	Non-susceptible to extended-spectrum cephalosporins and at least one carbapenem

Table 3: The clinical classification scheme for the classification of ESBLs.

1.8 CTX-M family of enzymes

CTX-M family is a rapidly growing group of enzymes belonging to ESBLs family (Baraniak *et al.*, 2002, Pitout, 2010). In fact, CTX-M family of enzymes has been shown to be the most successful type of ESBLs spreading around the globe (Canton and Coque, 2006, Cullik *et al.*, 2010, Pitout and Laupland, 2008, Rossolini *et al.*, 2008). The first case of CTX-M-β-lactamase was isolated in 1986 in Munich (Matsumoto *et al.*, 1988). In reference to the time line of ESBL descriptions (Figure 2), CTX-M outbreaks exploded during the 1990's (Bonnet, 2004, Chen *et al.*, 2005, Radice *et al.*, 2002, Rice, 2012, Tzouvelekis *et al.*, 2000). In the last decade, CTX-M enzymes were reported to be spreading world-wide causing hospital and community urinary tract infections (UTIs) being called ''the CTX-M pandemic'' (Ben-Ami *et al.*, 2006, Bonnet, 2004, Canton *et al.*, 2003, Canton and Coque, 2006, Chen *et al.*, 2005, Johnson *et al.*, 2010, Malloy and Campos, 2011, Nicolas-Chanoine *et al.*, 2008, Rogers *et al.*, 2011, Romero *et al.*, 2005). In addition, different reports

indicated the infiltration of CTX-M family of enzymes in community settings (Bush and Fisher, 2011, Canton and Coque, 2006, Coque *et al.*, 2008). In Kuwait, $bla_{\text{CTX-M-15}}$ gene was described in to be most prevalent in Kuwaiti hospital settings (Al Hashem *et al.*, 2011, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Coque *et al.*, 2008, Dashti *et al.*, 2010b, Ensor *et al.*, 2009, Jamal *et al.*, 2010, Rotimi *et al.*, 2008). Additionally, $bla_{\text{CTX-M-14}}$ was reported in Kuwait by Al-Hashem *et al.* (2011).

Moreover, different reports described the detection of some CTX-M family members in pets, farm animals, food products, and waste disposals (Brisse and Duijkeren, 2005, Frye and Fedorka-Cray, 2007, Geser *et al.*, 2012, Ho *et al.*, 2010, Jouini *et al.*, 2007, Kojima *et al.*, 2005, Li *et al.*, 2010, Pitout *et al.*, 2005, Romero *et al.*, 2005, Sun *et al.*, 2010, Tian *et al.*, 2012, Zheng *et al.*, 2012). The most successful family member of the CTX-M-ESBLs is known to be CTX-M-15 enzyme (Boyd *et al.*, 2004, Bush and Fisher, 2011, Canton *et al.*, 2008, Hawkey, 2008b). The spread of CTX-M-15 is mainly due to the spread of epidemic clones (such as ST131), or due to horizontal transfer plasmids carrying this gene (Pitout, 2010). The accumulating numbers of reports indicating the wide-spread of $bla_{\text{CTX-M-15}}$ gene. Also, points out the importance of studying this gene and possible causes for its spread.

1.9 Definition of CTX-M enzymes

The CTX-Ms are a heterogeneous family of enzymes that have earned this designation due to their resistance pattern to cefotaxime and to ceftazidime to a milder extent (Canton and Coque, 2006, Cartelle *et al.*, 2004). Nevertheless, with

their continual diversification their hydrolytic activity towards ceftazidime is compromised (Bush, 2010). Certain members of the CTX-M family have been reported to show enhanced efficiency for ceftazidime hydrolysis, such as $bla_{\text{CTX-M-15}}$ (Bush, 2010, Paterson and Bonomo, 2005, Pitout, 2010, Poirel *et al.*, 2002a). Moreover, CTX-M-ESBLs retain susceptibility to tazobactam and clavulanate inhibitors rather than sulbactam as β-lactamase inhibitors (Bush, 2010, Chroma and Kolar, 2010). CTX-M family of enzymes are of molecular class A (Ambler *et al.*, 1991, Hall and Barlow, 2005), functional group 2be (Bush *et al.*, 1995, Bush and Jacoby, 2010), and $ESBL_A$ (Giske *et al.*, 2009). Despite the fact that CTX-M-ESBLs are categorized into Ambler class A with TEM and SHV, they are not closely related to those two families (Bradford, 2001). With the Ambler classification, CTX-Ms share serine as their active site (same as TEM and SHV-ESBLs). In addition, key amino acids in CTX-Ms sequences at certain positions were reported to play a crucial role in the hydrolytic activity of CTX-M-β-lactamases. These amino acids are; Asn104, Asn132, Ser237, and Asp240 (Bonnet, 2004, Perez *et al.*, 2007). Mutations around these key amino acids may cause specific interactions leading to improved activity against ceftazidime and cefotaxime (Perez *et al.*, 2007).

1.10 Classification and origin of CTX-Ms

The suggested hypothesis of the origin of $bla_{\text{CTX-M}}$ genes is that they were naturally produced from chromosomal *bla* genes of environmental *Kluyvera* spp. (Canton *et al.*, 2008, Canton *et al.*, 2012, Eisner *et al.*, 2006). *Kluyvera* spp. are known to be

present in human intestines as normal microbes but with low bacterial counts. They are also considered to be saprophytic opportunistic pathogens (Farmer *et al.*, 1981). Moreover, these species have rarely been reported from clinical specimens. Less likely, they have been isolated from urine and skin tissues (Sarria *et al.*, 2001). Most reports of *Kluyvera* spp. are from environment (such as water, soil, and sewage) (Decousser *et al.*, 2001, Farmer *et al.*, 1981, Humeniuk *et al.*, 2002). It is believed that some $bla_{CTX\text{-}M}$ genes originated by means of mobilization from those chromosomal *bla* genes (Canton *et al.*, 2012). This hypothesis is supported by high amino acid similarities of CTX-M-type β-lactamases to chromosomal β-lactamase *Kluyvera* spp. genes (Paterson and Bonomo, 2005). In addition, CTX-M-β-lactamase genes are not closely related to TEM or SHV families, indicating different progenitors (Humeniuk *et al.*, 2002). Moreover, the hypothesis of the origin of CTX-M-ESBLs can also be supported by their horizontal mobilization from *Kluyvera* spp. to *Enterobacteriaceaeae* and non-pathogenic bacteria (Novais *et al.*, 2010, Rodriguez *et al.*, 2004). In comparison with TEM and SHV-ESBL, CTX-Ms have shown to express high rate of mobilization and reside on various genetic grounds (Barlow *et al.*, 2008). The CTX-M genes are thought to be captured from *Kluyvera* spp. and mobilized to other bacteria by mobile genetic elements (MGE) (such as IS*Ecp1*) (Pitout, 2010, Poirel *et al.*, 2005).

In terms of classification, CTX-M family of enzymes is diverged into five evolutionary groups based on their amino acid sequences homology. Namely, the described sub-groups are; CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, CTX-M-9 group, and CTX-M-25 group (Baraniak *et al.*, 2002, Boyd *et al.*, 2004, Cartelle *et al.*, 2004,

Dutour *et al.*, 2002). Interestingly, a recent study showed that CTX-M-74 and CTX-M-75, members of CTX-M-2 subgroup, could be classified into a sixth distinct CTX-M subgroup (Stepanova *et al.*, 2008). It is described that the members of each subgroup share > 95% similarity based on their amino acid sequence similarities. On the other hand, the members of each distinctive group utilize ≤ 90% similarity with the members of other CTX-M sub-groups (Bonnet, 2004, Pitout and Laupland, 2008, Rossolini *et al.*, 2008). Up to date, ninety-eight CTX-M variants have been curated by George Jacoby and Dr Karen Bush through the website (www.lahey.org/studies/webt.asp - last accessed (15th August 2012). The members of the each sub-group are described in Table 4. The CTX-M subfamilies are also represented in a dendogram in Figure 4. Previous propositions indicated that the possible progenitor of some CTX-M-1 group of enzymes is bla_{KLUC-1} and bla_{KLUC-2} from *Kluyvera cryocrscenes* (*K. cryocrscenes*) (Poirel *et al.*, 2008). Nevertheless, a recent study indicated that the real ancestor of this CTX-M subgroup might still be undetermined (Novais *et al.*, 2010). Other studies suggested that the probable natural parent of CTX-M-2 subgroup is bla_{KLUA-1} from *Kluyvera ascorbata* (*K. ascorbata*) based on the high homology between both genes (Canton and Coque, 2006, Edelstein *et al.*, 2003, Poirel *et al.*, 2008, Rodriguez *et al.*, 2004, Saladin *et al.*, 2002).

CTX-M sub-family	CTX-M family members
CTX-M-1 cluster	CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-11, CTX-M-12, CTX-M-15, CTX-M-22, CTX-M-28, CTX-M-29, CTX-M-30, CTX-M-32, CTX-M-33, CTX-M-34, CTX-M-36, CTX-M-37, CTX-M-42, CTX-M-52, CTX-M-53, CTX-M-54, CTX-M-55, CTX-M-57, CTX-M-58, CTX-M-60, CTX-M-61, CTX-M-62, CTX-M-64, CTX-M-66, CTX-M-68, CTX-M-69, CTX-M-71, CTX-M-79, CTX-M-82, CTX-M-89, CTX-M-101, CTX-M-107, CTX-M-108, CTX-M-109, CTX-M-114, CTX-M-116, CTX-M-117, and CTX-M-123.
CTX-M-2 cluster	CTX-M-2, CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20, CTX-M-31, CTX-M-35, CTX-M-43, CTX-M-56, CTX-M-59, CTX-M-74, CTX-M-75, CTX-M-92, CTX-M-97.
CTX-M-8 cluster	CTX-M-8, CTX-M-40, and CTX-M-63.
CTX-M-9 cluster	CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-18, CTX-M-19, CTX-M-21, CTX-M-24, CTX-M-27, CTX-M-38, CTX-M-51, CTX-M-65, CTX-M-67, CTX-M-83, CTX-M-84, CTX-M-85, CTX-M-86, CTX-M-90, CTX-M-98, CTX-M-99, CTX-M-102, CTX-M-104, CTX-M-105, CTX-M-106, CTX-M-110, CTX-M-111, CTX-M-112, CTX-M-113, CTX-M-121, CTX-M-122, and CTX-M-126.
CTX-M-25 cluster	CTX-M-25, CTX-M-26, CTX-M-39, CTX-M-41, 91, and CTX-M-94.

Table 4: CTX-M sub-groups and members.

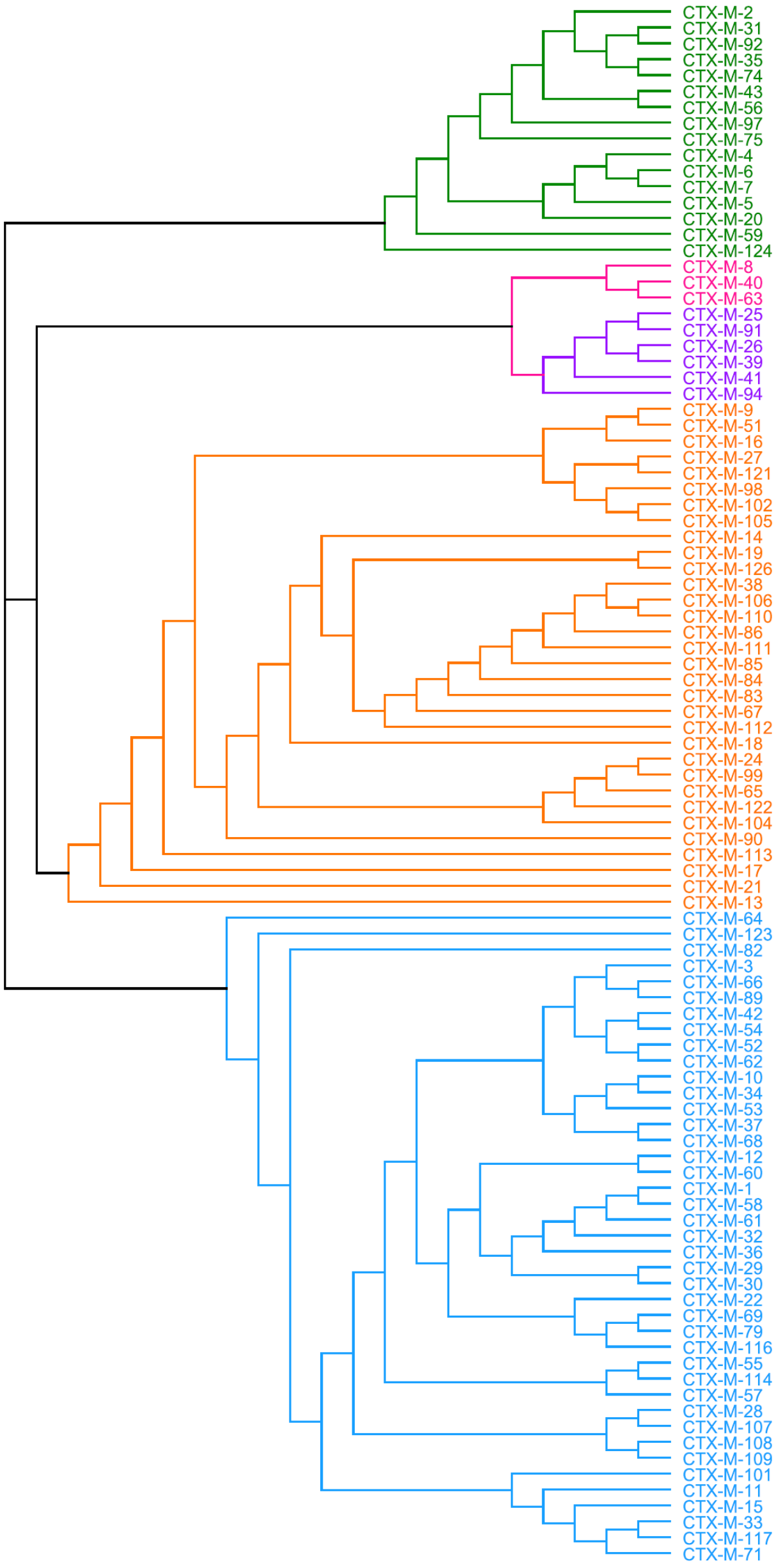


Figure 4: CTX-M family. This figure was constructed with Genious software, Version 5.6.4. Using cost matrix with 65% similarity, open gap penalty of 12, and gap extension penalty of 3. The nucleotide sequences of the provided CTX-Ms were aligned by the global alignment with free ends gaps and the genetic model of Jukes-Cantor. The tree build-up method was Neighbour joining. CTX-M-1 cluster is highlighted with light blue colour, CTX-M-2 group with green colour, CTX-M-8 cluster with pink colour, CTX-M-9 group with orange colour, and CTX-M-25 with purple colour.

Poirel *et al* (2002b) and Edelsteins *et al* (2003) suggested that the potential natural producer of CTX-M-8 subgroup is bla_{KLUG-1} of *Kluyvera georgiana* (*K. georgiana*). It was proved that the chromosomal bla_{KLUG-1} shares 83-88% amino acid identity with $bla_{CTX-M-8}$ (Poirel *et al.*, 2002b). On the other hand, Olson has identified *K. georgiana* as a possible progenitor of CTX-M-9 subgroup (Olson *et al.*, 2005). In addition, *K. georgiana* is also thought to be the probable ancestor of CTX-M-25 subgroup (Rodriguez *et al.*, 2010). Notably, two of *K. georgiana* chromosomal genes were proposed to be the possible precursors of two clusters of CTX-M enzymes. On the other hand, CTX-M-1 cluster is related to *K. ascorbata* and *K. cryocrscenes* (Canton *et al.*, 2012, Decousser *et al.*, 2001, Rodriguez *et al.*, 2004). This finding suggests the presence of other evolutionary trajectories (Canton *et al.*, 2012).

1.11 Evolution of CTX-M-βlactamases

The explosive reporting of the global un-controlled antibiotic resistance (AR) is the result of evolutionary processes and microbial variations (Martinez *et al.*, 2007). The investigation of the past mutational pathways and prediction of the hypothetical evolutionary trajectories and scenarios of AR-genes could enable scientists to predict future trends of AR (Martinez *et al.*, 2007, Novais *et al.*, 2010).

Long ago, the evolution of AR of bacterial strains was present in nature. This was considered as a self defence mechanism against natural antibiotics (Pallecchi *et al.*, 2008). Nevertheless, this mode of resistance evolved rapidly and invaded the

clinical settings (Gniadkowski, 2008). Seemingly, many factors manipulated the evolution of AR-genes, some of which are; the large population and the earlier diversity of the microbial species encoding the AR-genes (Gniadkowski, 2008, Martinez *et al.*, 2007), the strong selective pressure exerted by the extensive use of antimicrobial drugs (Gniadkowski, 2008, Pallecchi *et al.*, 2008), the presence of mutational hot-spots (Gniadkowski, 2008), and extensive HGT (Suzuki *et al.*, 2010).

The evolution of β-lactamases presumably occurred from the combat with β-lactam antibiotics present in nature produced by *Streptomyces*, *Lysobacter*, filamentous fungi, or *Acremonium* (Bassetti *et al.*, 2011a, Bassetti *et al.*, 2011b). However, soon after the introduction of β-lactam antibiotics into the clinical practice and their extensive use, β-lactamases evolved rapidly limiting treatment options (Bassetti *et al.*, 2011a, Bassetti *et al.*, 2011b, Hawkey, 2008a, Smet *et al.*, 2010). Of ESBLs, class A are considered to be the largest diversified group (Gniadkowski, 2008). In comparison with bla_{TEM} and bla_{SHV}, CTX-M β-lactamases were the most successful family in terms of distribution, prevalence, dissemination, and evolution (Canton *et al.*, 2012). Clearly, different factors drove the evolutionary pathways of these enzymes to the presence of various CTX-M alleles and with more efficient phenotypic profiles (Cartelle *et al.*, 2004, Novais *et al.*, 2008, Poelwijk *et al.*, 2007, Poirel *et al.*, 2002b, Weinreich *et al.*, 2006). CTX-M-1 sub-group is considered to be the most diversified sub-group (Novais *et al.*, 2010). This is indicated by the increased number of variants of this group described in the last few years. Most

importantly, these evolutionary processes occurred in a considerable short period of time (Canton *et al.*, 2012, Novais *et al.*, 2010). The resulting alleles proved to express an enhanced resistance phenotype with more powerful hydrolytic activity towards cefotaxime and ceftazidime (Mamlouk *et al.*, 2006, Novais *et al.*, 2010, Poelwijk *et al.*, 2007, Perez-Llarena *et al.*, 2011).

The first evolutionary phase of CTX-M family of enzymes led to their diversification from *Kluyvera* spp. into five sub-groups. Then, the evolutionary processes directed the generation of members of each sub-group (Gniadkowski, 2008). The later diversification was associated with the extensive use of cefotaxime and ceftazidime acting as a selective pressure directing the rapid increase in the derivatives of CTX-M-3 gene as a member of CTX-M-1 group (Biondi *et al.*, 2011, Hawkey, 2008b, Hawkey and Jones, 2009, Livermore, 2003, Rice, 2009). The evolutionary hot-spots found in both nature and directed mutagenesis of CTX-M enzymes have indicated changes are most prevalent at positions Asn104, Asn132, Ser237, and Asp240 (Gniadkowski, 2008, Perez *et al.*, 2007). Other key factors involved in the rapid evolution of CTX-M family of enzymes is HGT by MGE (Boto, 2010, Choi and Kim, 2007, Ensor *et al.*, 2009, Gangoue-Pieboji *et al.*, 2005, Frost *et al.*, 2005, Smet *et al.*, 2010, Suzuki *et al.*, 2010). HGT is a mean of the acquisition of new genes and functions (Boto, 2010). The impact of HGT on evolutionary process is still controversial. Regardless, some studies proved that HGT affects evolution act as a driving force in the evolution of CTX-M enzymes (Hawkey and Jones, 2009, Pallecchi

et al., 2008). The acquisition of CTX-M genes is mainly driven by the action of insertion elements (Frost *et al.*, 2005). These MGE are well documented in literature to be responsible for the mobilization and transposition of CTX-M genes (Lartigue *et al.*, 2004, Diestra *et al.*, 2009).

Insertion elements are compact DNA sequences that are relatively small in size (less than 2.5 Kb in length) (Mahillon *et al.*, 1999). These DNA segments encode genes for their function. The main function of these elements is to transpose and translocate between genomes (Mahillon *et al.*, 1999). With the ability of these small sequences for transposition, they promote genetic rearrangements (such as deletions, inversions, or fusion of replicons). Hence, insertion sequences (IS) elements play a major role in the plasticity of the prokaryotic genomes (Mahillon and Chandler, 1998). One of the most common IS elements that was highly associated with certain CTX-M family member IS*Ecp1* with members of CTX-M-1 group (Lartigue *et al.*, 2004).

Eventually, all of these factors together increased the rapidity of the evolutionary processes of CTX-M enzymes and led to extensive reports in literature as well as increased activity towards cefotaxime and ceftazidime (Gniadkowski, 2008, Novais *et al.*, 2010).

1.12 Spread of CTX-Ms in *Enterobacteriaceaeae*

CTX-M-β-lactamases are increasingly being reported in *Enterobacteriaceaeae* family (Boyd *et al.*, 2004). Many reports indicated the increased prevalence of bla_{CTX-M} genes with *Enterobacteriaceaeae* (Bassetti *et al.*, 2011b, Bassetti *et al.*, 2011a, Bush, 2010, Dutour *et al.*, 2002, Eckert *et al.*, 2006, Gangoue-Pieboji *et al.*, 2005, Karisik *et al.*, 2006, Lavollay *et al.*, 2006, Naseer *et al.*, 2009, Woodford *et al.*, 2006). Some members of these family carrying bla_{CTX-M} genes are involved in nosocomial and community infections (Karisik *et al.*, 2006). Interestingly, bla_{CTX-M} genes are being increasingly reported to be associated with *E. coli* especially from UTIs (Ho *et al.*, 2010, Jouini *et al.*, 2007, Kanj *et al.*, 2008, Oteo *et al.*, 2010, Peirano and Pitout, 2010, Pitout, 2010, Rice, 2012). Moreover, *K. pneumoniae* encoding bla_{CTX-M} genes is globally reported to be causing serious hospital-acquired infections (Bagattini *et al.*, 2006, Brisse *et al.*, 2004, Brisse and Verhoef, 2001, Diancourt *et al.*, 2005, Wang *et al.*, 2009, Shi *et al.*, 2009). A serious attention should be given to the spread of CTX-M enzymes in *E. coli* and *K. pneumoniae*, in particular $bla_{CTX-M-15}$ which was witnessed from different hospital around the world (Coelho *et al.*, 2010, Karisik *et al.*, 2006, Lavollay *et al.*, 2006, Malloy and Campos, 2011, Mamlouk *et al.*, 2006, Nicolas-Chanoine *et al.*, 2008).

1.13 Spread of CTX-Ms in the world, in the Middle East, and in Kuwait

The presence of a certain member of CTX-M family is not restricted to a specific geographical area; rather variable CTX-Ms members are present in different geographical areas (Jean and Hsueh, 2011). Earlier studies pointed out the presence of certain CTX-M variants to be related with specific geographical regions (Hawkey and Jones, 2009). Admittedly, there is a dominant CTX-M gene in every country, but this does not necessarily indicate that this specific gene is restricted to one area than other areas as suggested by Hawkey (Hawkey and Jones, 2009). Notably, CTX-M-1 and CTX-M-9 group members are the most successful members in terms of spread. In particular CTX-M-15 and CTX-M-14 respectively (Malloy and Campos, 2011). Surveillance of recent studies allowed us to conclude that the latter two enzymes are most common and most likely to cause endemics in Asia, Europe, South America, and the United States (Hawkey and Jones, 2009). All studies agree of the widespread and dissemination of $bla_{\text{CTX-M-15}}$ gene in clinical and community settings causing a ''pandemic'' (Canton and Coque, 2006). CTX-M-15 continues to be the most commonly reported CTX-M enzyme in Europe, with the exception of Spain where CTX-M-14 is the most prevalent (Karisik *et al.*, 2006, Lartigue *et al.*, 2004, Malloy and Campos, 2011, Mamlouk *et al.*, 2006, Mena *et al.*, 2006, Menezes *et al.*, 2010, Nicolas-Chanoine *et al.*, 2008). Moreover, CTX-M-15 is the most prevalent enzyme in the United States and Africa (Lartigue *et al.*, 2004, Malloy and Campos, 2011, Menezes *et al.*, 2010, Peirano and Pitout, 2010). In addition, CTX-M-15 is the most prevalent enzyme in Asia (Jean and Hsueh, 2011, Lartigue *et al.*,

2004, Malloy and Campos, 2011, Menezes *et al.*, 2010, Nicolas-Chanoine *et al.*, 2008, Novais *et al.*, 2007). The distribution of $bla_{\text{CTX-M-15}}$ can be seen in Figure 5.

Figure 5: World map of the distribution of *bla*$_{\text{CTX-M-15}}$. The areas shaded in grey represent reports from countries with *bla*$_{\text{CTX-M-15}}$. Adopted from Pitout *et al* (2010).

In the Middle East, CTX-M-15 was reported to be the most common enzyme of CTX-M family (Jean and Hsueh, 2011, Lartigue *et al.*, 2004, Malloy and Campos, 2011, Menezes *et al.*, 2010, Nicolas-Chanoine *et al.*, 2008, Novais *et al.*, 2007). Unfortunately, lack of reports from some countries of the Middle East is observed from some countries like Cyprus, Jordan, Lebanon, Palestine, Qatar, Syria, and Yemen. It is of note that the reporting of CTX-M enzymes is less than required and small amount of information is maintained. The situation in the Middle East in terms of CTX-M prevalence should be studied more thoroughly. In Table 5, the most prevalent member of CTX-M family is reported from countries of the Middle East. However, $bla_{\text{CTX-M-15}}$ is noted to be the most common member of CTX-M family in the Middle East (Table 5).

Most of the reports from Kuwait described $bla_{\text{CTX-M-15}}$ to be the most common ESBL enzyme (Al Hashem *et al.*, 2011, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Coque *et al.*, 2008, Dashti *et al.*, 2010b, Ensor *et al.*, 2009, Jamal *et al.*, 2010, Rotimi *et al.*, 2008). However, one report described indicated the presence of $bla_{\text{CTX-M-14}}$ (Al Hashem *et al.*, 2011). Nevertheless, the description of $bla_{\text{CTX-M-15}}$ from Kuwaiti hospitals is thought to be preliminary and a lot has been left undiscovered. A map illustrating the most common CTX-M enzyme in each country in the Middle East is shown in Figure 6.

Country	CTX-M member	Reference
Bahrain	No reports available	—
Cyprus	No reports available	—
Egypt	*bla*$_{CTX-M-15}$	(Ahmed and Shimamoto, 2011, Fam *et al.*, 2011, Khalaf *et al.*, 2009, Tham *et al.*, 2010)
	bla$_{CTX-M-14}$	(AbdelGhani *et al.*, 2010, Khalaf *et al.*, 2009, Tham *et al.*, 2010)
	bla$_{CTX-M-2}$	(Hammad and Shimamoto, 2011)
	bla$_{CTX-M-27}$	(Mohamed Al-Agamy *et al.*, 2006)
Iran	*bla*$_{CTX-M-15}$	(Feizabadi *et al.*, 2010a, Feizabadi *et al.*, 2010b, Hamidian *et al.*, 2009, Ranjbar *et al.*, 2010, Tajbakhsh *et al.*, 2012)
Iraq	*bla*$_{CTX-M-15}$	(Huang *et al.*, 2012, Pfeifer *et al.*, 2009)
Jordan	No reports available	—
Kuwait	*bla*$_{CTX-M-15}$	(Al Hashem *et al.*, 2011, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Coque *et al.*, 2008, Dashti *et al.*, 2010b, Ensor *et al.*, 2009, Jamal *et al.*, 2010, Rotimi *et al.*, 2008)
	bla$_{CTX-M-14}$	(Al Hashem *et al.*, 2011)
Lebanon	*bla*$_{CTX-M-15}$	(Kanj *et al.*, 2008, Matar *et al.*, 2005, Matar *et al.*, 2007, Matar *et al.*, 2008, Matar *et al.*, 2010, Moubareck *et al.*, 2005a, Moubareck *et al.*, 2005b, Sabra *et al.*, 2009)
Oman	*bla*$_{CTX-M-15}$	(Poirel *et al.*, 2011)
Palestine	No reports available	—
Qatar	No reports available	—
Syria	No reports available	—
Turkey	*bla*$_{CTX-M-15}$	(Aktas *et al.*, 2008, Aktas *et al.*, 2012, Azap *et al.*, 2010, Carrer *et al.*, 2008, Celik *et al.*, 2010, Gonullu *et al.*, 2008, Kilic *et al.*, 2011, Nazik *et al.*, 2008, Randall *et al.*, 2011, Yumuk *et al.*, 2008, Weill *et al.*, 2004)
	bla$_{CTX-M-3}$	(Acikgoz *et al.*, 2008, Agin *et al.*, 2011, Bahar *et al.*, 2006, Galimand *et al.*, 2005, Nazik *et al.*, 2008, Samuelsen *et al.*, 2009)
	bla$_{CTX-M-1}$	(Wasyl *et al.*, 2012)
United Arab Emirates	*bla*$_{CTX-M-15}$	(Sonnevend *et al.*, 2006, Rotimi *et al.*, 2008)
Yemen	No reports available	—

Table 5: Most common CTX-M member reported in countries of the Middle East.

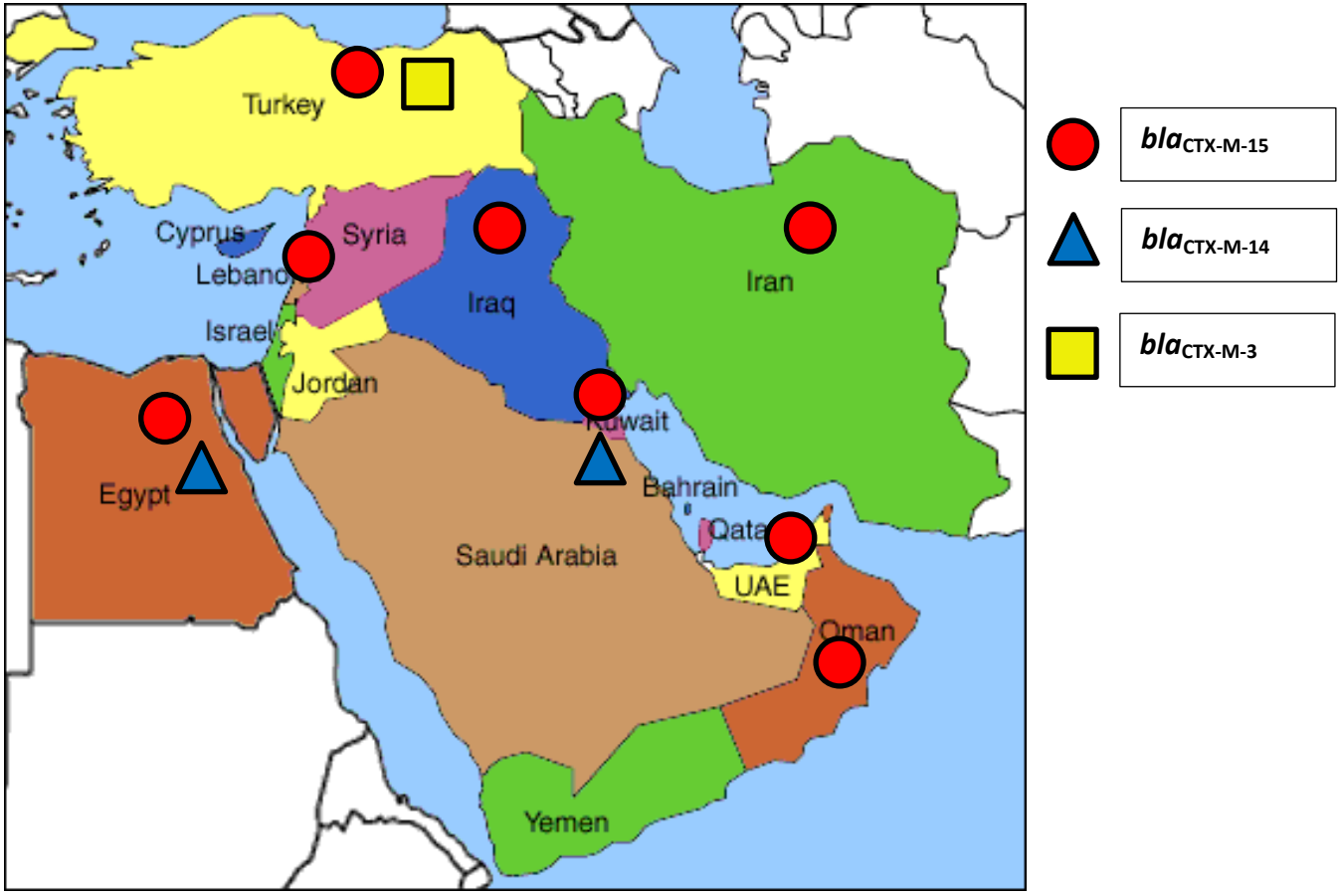


Figure 6: Middle East map and the prevalence of most common CTX-M enzymes.

In Kuwait, there are 13 governmental hospitals; mainly there is one in each district though more than one hospital can be located in the same district as they may serve specialized conditions (e.g. hospitals for maternity cases, hospitals specialized in chest infections or hospitals dealing with infectious diseases only). There are however, five main districts in Kuwait. These are Hawali district, Al-Kuwait district, Al-Farwaniya district, Al-Jahra district, and Mubarak Al-Kabeer with Al-Ahmadi district. In Al-Jahra district most of the specialized hospitals serving as an integrated medical area and a part of that district. Usually, cases of emergency and serious illnesses are treated in one of the five main hospitals (Amiri, Mubarak AlKabeer, Adan, Farwaniya, and Al-Jahra hospitals) and specialized conditions are referred to one of the specialized hospitals in the Al-Jahra district, depending on the diagnosis (e.g. patients diagnosed with HIV or tuberculosis are referred to Infectious diseases hospital).

The Al-Amiri hospital is in the Al-Kuwait district, serving populations only living in that area. While, the Infectious Diseases, Maternity, and Ibn-Sina hospitals, are all located in the Al-Jahra district, serve populations from different areas in Kuwait as they are specialized hospitals for infectious diseases, maternity and neonate cases, bone and rheumatic disorders, respectively. The KOC hospital is in the private sector, only serving workers in the Kuwait Oil Company and relatives of those workers. The populations being served in the latter hospital are heterogeneous and not restricted to a certain districts. Locations of the hospitals in districts are shown in Figure 7. These hospitals are open to all nationals living and working in Kuwait.

Previous reports indicated that the most common *bla*$_{CTX-M}$ gene in Kuwaiti hospitals is *bla*$_{CTX-M-15}$ (Rotimi *et al.*, 2008, Ensor *et al.*, 2009, Dashti *et al.*, 2010b, Dashti *et al.*, 2010a, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Al Hashem *et al.*, 2011). The second most common *bla*$_{CTX-M}$ gene in Kuwaiti hospitals is *bla*$_{CTX-M-14}$ (Al Hashem *et al.*, 2011). The hospitals from which isolates were collected in earlier reports were Al-Adan, Alamiri, farwaniya, Ibn-sina, al-Jahra, maternity, Mubarak, and Al sabah hospitals. However, previous studies did not obtain any information from the infectious diseases hospital and Kuwait oil company hospitals. The hospitals from which information regarding the prevalence of *bla*$_{CTX-M}$ enzymes in Kuwait are shown in Figure 7.

	Hospital
1	Amiri
2	Infectious Diseases
3	Maternity
4	Ibn-Sina
5	KOC
6	Adan hospital
7	Aljahra hospital
8	Alsabah hospital
9	Mubarak al-kabeer hospital

Figure 7: Locations of hospitals in Kuwait with different Kuwait districts.

2 Objectives

1. To detect the spread of CTX-M-β-lactamases in Kuwaiti hospitals from which the strains were collected.

2. To detect the resistance profiles of the collected isolates and determine MDR patterns.

3. To detect the genotypic relatedness using PFGE among the collected isolates and possible spread of a certain clone.

4. To detect the most common member of CTX-M family of enzymes.

5. To provide analysis of the genetic environment of $bla_{\text{CTX-M}}$ genes.

6. To provide structural analysis of the reported CTX-M enzymes and mutational effects.

3 Materials and Methods

Sample collection and storage

One hundred and seven isolates of cefotaxime-resistant *E. coli* (84) and *K. pneumoniae* (23) were collected between 2006 and 2010 from five hospitals in Kuwait; the Al-Amiri hospital (A), the Ibn-sina hospital (IB), the Infectious Diseases hospital (ID), the Kuwait Oil Company hospital (KOC) and the Maternity hospital (M). The isolates were transported from the hospital laboratory to the university research laboratories of Kuwait University onto Nutrient agar plates (Oxoid Ltd, Basingstoke, UK) after overnight incubation at 37^{o}C. All of the transported strains were stored at -70^{o}C. All the strains used in this study, 84 (78.50%) *E. coli* and 23 (21.49%) *K. pneumoniae* along with their clinical data are listed in Table 6**.** The sources of the isolates were as follows; 65 (60.74%) from urine, 17 (15.88%) from wound swabs, 8 (7.47%) from vaginal swabs, 5 (4.67%) from Endotracheal tip, 3 (2.80%) from blood cultures, 3 (2.80%) from sputum, 3 (2.80%) from rectal swabs, 1 (0.9%) from body fluids, 1 (0.9%) catheter tips and 1 (0.9%) pus. Summary of the sources of the isolates can be seen in Table 7.

Number	Hospital	Strain	Specimen Source	Gender
1	M	*E. coli*	Urine	F
2	M	*E. coli*	Urine	F
3	M	*E. coli*	Urine	F
4	M	*E. coli*	Endotracheal Tip	F
5	M	*K. pneumoniae*	Vaginal Swab	F
6	M	*E. coli*	Urine	F
7	M	*K. pneumoniae*	Wound Swab	F
8	M	*E. coli*	Urine	F
9	M	*K. pneumoniae*	Wound Swab	F
10	M	*E. coli*	Urine	F
11	M	*E. coli*	Urine	F
12	M	*E. coli*	Vaginal Swab	F
13	M	*E. coli*	Wound Swab	F
14	M	*E. coli*	Urine	F
15	M	*E. coli*	Wound Swab	F
16	M	*K. pneumoniae*	Blood Culture	F
17	M	*E. coli*	Urine	F
18	M	*K. pneumoniae*	Rectal Swab	F
19	M	*K. pneumoniae*	Vaginal Swab	F
20	M	*K. pneumoniae*	Urine	F
21	M	*E. coli*	Vaginal Swab	F
22	M	*E. coli*	Urine	F
23	M	*E. coli*	Endotracheal Tip	F
24	M	*E. coli*	Urine	F
25	M	*E. coli*	Urine	F
26	M	*E. coli*	Wound swab	F
27	M	*E. coli*	Urine	F
28	M	*K. pneumoniae*	Urine	F

Number	Hospital	Strain	Specimen Source	Gender
29	M	*K. pneumoniae*	Urine	F
30	M	*E. coli*	Wound Swab	F
31	M	*K. pneumoniae*	Urine	F
32	M	*K. pneumoniae*	Endotracheal Tip	F
33	M	*E. coli*	Urine	F
34	M	*E. coli*	Urine	F
35	M	*E. coli*	Vaginal Swab	F
36	M	*E. coli*	Urine	F
37	M	*K. pneumoniae*	Blood Culture	F
38	M	*K. pneumoniae*	Blood Culture	F
39	M	*E. coli*	Urine	F
40	M	*E. coli*	Vaginal Swab	F
41	M	*K. pneumoniae*	Vaginal Swab	F
42	M	*E. coli*	Vaginal Swab	F
43	M	*K. pneumoniae*	Urine	F
44	M	*K. pneumoniae*	Rectal Swab	F
45	M	*K. pneumoniae*	Rectal Swab	F
46	M	*K. pneumoniae*	Urine	F
47	M	*E. coli*	Wound Swab	F
48	M	*E. coli*	Wound Swab	F
49	M	*E. coli*	Urine	F
50	M	*E. coli*	Pus	F
51	M	*E. coli*	Urine	F
52	K	*E. coli*	Urine	F
53	K	*E. coli*	Urine	M
54	K	*E. coli*	Urine	F
55	K	*E. coli*	Urine	F
56	K	*E. coli*	Urine	M

Number	Hospital	Strain	Specimen Source	Gender
57	K	*E. coli*	Urine	F
58	K	*E. coli*	Urine	F
59	K	*E. coli*	Urine	F
60	K	*E. coli*	Wound Swab	F
61	K	*E. coli*	Catheter Tip	M
62	K	*E. coli*	Urine	F
63	K	*E. coli*	Urine	F
64	K	*E. coli*	Urine	F
65	K	*E. coli*	Urine	M
66	K	*E. coli*	Urine	F
67	K	*E. coli*	Urine	F
68	K	*E. coli*	Urine	F
69	K	*E. coli*	Urine	F
70	ID	*E. coli*	Urine	F
71	ID	*E. coli*	Wound Swab	F
72	ID	*E. coli*	Wound Swab	F
73	ID	*E. coli*	Wound Swab	F
74	ID	*E. coli*	Urine	F
75	ID	*E. coli*	Urine	F
76	ID	*E. coli*	Urine	M
77	ID	*E. coli*	Wound Swab	M
78	ID	*E. coli*	Urine	M
79	ID	*E. coli*	Wound Swab	M
80	ID	*K. pneumoniae*	Urine	M
81	ID	*E. coli*	Urine	F
82	ID	*K. pneumoniae*	Urine	M
83	ID	*E. coli*	Wound Swab	F
84	ID	*E. coli*	Wound Swab	F

Number	Hospital	Strain	Specimen Source	Gender
85	ID	*K. pneumoniae*	Urine	F
86	ID	*E. coli*	Urine	M
87	ID	*E. coli*	Urine	F
88	A	*K. pneumoniae*	Urine	F
89	A	*K. pneumoniae*	Urine	F
90	A	*E. coli*	Urine	F
91	A	*E. coli*	Urine	M
92	A	*E. coli*	Urine	M
93	A	*E. coli*	Urine	F
94	A	*E. coli*	Urine	F
95	A	*E. coli*	Urine	F
96	A	*E. coli*	Urine	M
97	A	*E. coli*	Urine	M
98	A	*E. coli*	Urine	F
99	A	*E. coli*	Urine	F
100	IB	*E. coli*	Urine	F
101	IB	*E. coli*	Sputum	M
102	IB	*E. coli*	Drain Fluid	M
103	IB	*E. coli*	Sputum	M
104	IB	*E. coli*	Sputum	M
105	IB	*E. coli*	Wound Swab	M
106	IB	*E. coli*	Endotracheal Tip	F
107	IB	*E. coli*	Endotracheal Tip	F

Table 6: Isolates and clinical data. A refers to the Al-Amiri hospital, IB refers to the Ibn-Sina hospital, ID refers to the Infectious Diseases hospital, KOC refers to the Kuwait Oil Company hospital, and M refers to the Maternity hospital.

Source	No. (%) of patients
Blood Culture	3 (2.80%)
Catheter Tip	1 (0.90%)
Drain Fluid	1 (0.90%)
Endotracheal Tip	5 (4.67%)
Pus	1 (0.90%)
Rectal Swab	3 (2.80%)
Sputum	3 (2.80%)
Urine	65 (60.74%)
Vaginal Swab	8 (7.47%)
Wound Swab	17 (15.88%)

Table 7: Sources of samples.

3.1 Determination of minimum inhibitory concentrations (MICs)

All isolates were tested for their susceptibility to four different classes of antibiotics to detect their spectrum of activity. The antibiotics used were; gentamicin (aminoglycoside); ertapenem, imipenem and meropenem (carbapenems); cefepime, cefoxitin, cefotaxime, and ceftazidime (cephalosporins); and ciprofloxacin as fluoroquinolone. The MICs were determined by double dilutions agar method described by the BSAC (Andrews, 2010). The breakpoints of the antibiotics used were in reference to Andrews (2012)(Andrews, 2012). All agars and broth used in MIC determination were from Oxoid (Basingstoke, Hants). The normal saline used was prepared from 0.85% NaCl with sterile distilled water. The control strains used

were *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Staphylococcus aureus* (*S. aureus*) NCTC 6571. The antibiotic classes, breakpoints and manufacturers are listed in Table 8.

Name of the antimicrobial agent	Class of the antimicrobial agents	Antimicrobial agent break point	Manufacturer
Cefepime	Fourth generation cephalosporin	4 mg/L	Sigma-Aldrich
Cefotaxime	Third generation cephalosporin	2 mg/L	Sigma-Aldrich
Cefoxitin	Second generation cephalosporin	8 mg/L	Sigma-Aldrich
Ceftazidime	Third generation cephalosporin	4 mg/L	Sigma-Aldrich
Ciprofloxacin	second generation flouroquinolone	1 mg/L	(Bayer AG, Germany)
Gentamicin	Aminoglycoside	4 mg/L	Sigma-Aldrich
Imipenem	Carbapenem	8 mg/L	(Merck Sharpe Dohme, Rahway, NJ, USA)
Meropenem	Carbapenem	8 mg/L	(ASTRA Zeneca, Loughborough, UK)
Ertapenem	Carbapenem	1 mg/L	(Merck Sharpe Dohme, Rahway, NJ, USA)
Gentamicin	Aminoglycoside	4mg/L	Sigma-Aldrich

Table 8: Antimicrobial agents name, class, breakpoint and manufacturer.

3.2 Phenotypic and molecular identification of *bla*$_{CTX-M}$ genes

For ESBL screening, the strains were identified phenotypically with Vitek 2 system and Double Disc Diffusion (DDD) method. Screening of ESBL production was maintained with Vitek 2 system (Bio Merieux, Marcy L'Etoile, France) (Paterson and Bonomo, 2005). Then, confirmation of ESBL-production was carried out by disc diffusion with cefotaxime and clavulanic acid (Oxoid) according to CLSI criteria (National Committee for Clinical Laboratory Standards., Clinical and Laboratory Standards Institute., 2005) (Malloy and Campos, 2011). The DDD method, uses two discs containing cefotaxime and ceftazidime Sigma-Aldrich Company (Poole, UK Ltd) of 30 µg that are placed onto two different plates of Iso-sensitest agar (IST) agar (Oxoid) inoculated with the strain. Then, the plates are inoculated at 37^{o}C overnight with the presence of clavulanate disc. The presence of an inhibition zone between cefotaxime or ceftazidime and clavulanate discs is indicative of ESBL production. CTX-M-producers will show a bigger zone with cefotaxime than ceftazidime (Clinical and Laboratory Standards Institute., 2005).

The genotypic identification of *bla*$_{CTX-M}$ genes was achieved using polymerase chain reaction (PCR). First, consensus primers were used for the detection of any of the *bla*$_{CTX-M}$ genes (Saladin *et al.*, 2002). Then, primers specific for each CTX-M groups were used (i.e. CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 groups). The annealing temperatures and product sizes of the primers are listed in Table 9. The primers used for CTX-M-1 group were designed using Primer3 software in an online

website (http://frodo.wi.mit.edu/primer3/input.htm - last accessed (15th August 2012) and manufactured by Invitrogen™, UK. For amplification, the strains were sub-cultured overnight at 37°C on MacConkey agar plates (Oxoid) and the DNA was extracted by boiling loop-full colonies in 100 µL sterile distilled water for 10 minutes. PCRs were performed in 25 µL volumes containing 3 µL of DNA, 2.5 µL of 5X Green Go Taq Flexi Buffer, 1.5 mM (2 µL) $MgCl_2$, 0.1 mM (1 µL) PCR nucleotide mix, 0.2 Units of *Taq*, and 0.1 mM (1 µL) of each primer (Promega, Southampton. UK). Distilled water was used as a diluent for all amplification reactions. PCR products were analysed on 1.4% w/v agarose gels (GenSieve LE agarose, Flowgen Bioscience, UK) with 1X Tris-acetic acid EDTA (TAE) loading buffer. The preparation of 1X TAE buffer was performed by diluting a stock 10XTAE buffer 1:10. The stock 10X TAE buffer was prepared from 3.72g EDTA, 11.4 ml glacial acetic acid (Biokeystone Co LLC, California, USA). The final volume of the buffer was rectified to 1L and the pH was adjusted to 8.0.

The marker used was 100bp ladder (New England Biolabs, UK). The gels were run for 45 minutes with 120 volts. Then, the gels were stained using 3X staining solution of gelRed dye (Biotium, Hayward, USA), which was prepared from a stock reagent of The 3X staining solution was prepared with 45 mL sterile distilled water, 5 mL of (1 M) NaCl, and 15 µL of the stock solution and kept at room temperature for storage and future use. After running, the gels were visualized under Ultra Violet transilluminator Bio-Rad Gel Doc 2000 (Bio-Rad, Hemel Hemstead, UK).

At this stage no sequencing for further identification of the CTX-M family members was done. Rather, the determination of the MICs was performed first to later selecting of strains with MDR pattern in reference to Zhanel *et al* (2008) criteria for defining MDR (Zhanel *et al.*, 2008).

Name	Nucleotide sequence	Size	Annealing temperature	Reference
CTX-M consensus	**F** 5'-SCSATGTGCAGYACCAGTAA-3' **R** 5'-CCGCRATATGRTTGGTGGTG-3'	504bp	52°C	(Saladin *et al.*, 2002) Corrected
CTX-M-1 group members	**F** 5'-CTGCGYCAGTTCACGCTSAT-'3 **R** 5'-CCGTYGGTGACGATTTTAGC-3'	854bp	62°C	Designed
CTX-M-2 group members	**F** 5'-GCGACCTGGTTAACTACAATCC-'3 **R** 5'-CGGTAGTATTGCCCTTAAGCC-'3	351bp	55°C	(Pitout *et al.*, 2004)
CTX-M-8 group members	**F** 5'-CGCTTTGCCATGTGCAGCACC-'3 **R** 5'-GCTCAGTACGATCGAGCC–'3	307bp	55°C	(Pitout *et al.*, 2004)
CTX-M-9 group members	**F** 5'-GCTGGAGAAAAGCAGCGGAG–'3 **R** 5'-GTAAGCTGACGCAACGTCTG– '3	474bp	62°C	(Pitout *et al.*, 2004)
CTX-M-25 group members	**F** 5'-GCACGATGACATTCGGG–'3 **R** 5'-AACCCACGATGTGGGTAGC– '3	327bp	52°C	(Woodford *et al.*, 2006)

Table 9: Primers used for the detection of *bla*$_{CTX-M}$ genes.

3.3 Pulsed-field gel electrophoresis (PFGE)

All of the isolates producing bla_{CTX-M} genes were genotyped using an enhanced rapid PFGE protocol that was modified from Durmaz (2009) method (see below). DNA fingerprinting profiles for the isolates were analysed digitally with BioNumerics software (Applied Maths, Gent, Belgium) to describe the clonal relationship among the collected isolates and to detect their epidemiologic relatedness.

Only subsets of isolates were typed by the Miranda (1996) method. Validation of the enhanced PFGE protocol was made by analysing and comparing the results of the subsets of isolates obtained from both procedures.

3.3.1 Prolonged PFGE method

The prolonged PFGE method was followed exactly as described previously without modifications (Miranda *et al.*, 1996). Isolates found to be identical with the enhanced rapid PFGE protocol were also genotyped with the traditional PFGE method. These isolates were 3 sets of identical *E. coli* strains and 3 sets of *K. pneumoniae* strains. Also, 30 isolates found to be different with the rapid PFGE method were genotyped with the prolonged PFGE protocol. These isolates were 30 *E. coli* strains sharing <85% similarity. The strains selected to be genotyped using both methods are listed in Table 10. Genotyping, both identical and different subsets of isolates, was done for validation purposes of the enhanced rapid method described in this study. The original method requires longer time than the enhanced shorter method to yield banding patterns for the strains. Lysis step requires two

days with two different buffers (i. e. lysis buffer and ESP buffer [0.4 M EDTA, 1% sodium lauroyl sarcosine, and 0.5 mg/ml of proteinase K (PK) (Sigma), pH 8.0]). This method is labour-intensive as there are different buffers used (PIV buffer, lysis buffer, ESP buffer, TE buffer, and TBE buffer) (Miranda *et al.*, 1996).

Strain number	strain
1-2	*E. coli*
21-22	*E. coli*
97-98	*E. coli*
32-28 and 37-38	*K. pneumoniae*
3	*E. coli*
4	*E. coli*
6	*E. coli*
11	*E. coli*
13	*E. coli*
14	*E. coli*
15	*E. coli*
17	*E. coli*
24	*E. coli*
30	*E. coli*
35	*E. coli*
36	*E. coli*
42	*E. coli*
47	*E. coli*
50	*E. coli*
51	*E. coli*
54	*E. coli*
55	*E. coli*
56	*E. coli*
57	*E. coli*
63	*E. coli*
64	*E. coli*
66	*E. coli*
75	*E. coli*
84	*E. coli*
86	*E. coli*
100	*E. coli*
101	*E. coli*
107	*E. coli*
105	*E. coli*

Table 10: Subset of strains selected to be typed by both methods. The strains highlighted in green are similar and identical.

3.3.2 Enhanced rapid PFGE method

The method used was modified from that of Durmaz (2009). There were three basic steps in which the method of Durmaz (2009) was modified; cell suspension preparation, cell lysis, and washing the agarose plugs.

Cell suspension preparation: Cell suspension was prepared by emulsifying 5-10 colonies of pure 18 hours bacterial cultures into 2 mL of 1X phosphate Buffered Saline (PBS) (Sigma-aldrich). The cell concentration was adjusted to absorbance value of 1 OD unit measured at 590 nm. The cell suspension was not vortexed to avoid cell breakage; rather it was gently inverted. Then, 2 mL of 1.6% certified megabase agarose (CMA) (Bio-Rad, Hemel Hempstead, UK) with 1% sodium dodecyl sulphate (SDS) (Fisher Scientific) was added without further mixing. About 500 µL of this mixture was dispensed into plug moulds (Bio-Rad) and kept at 4^{o}C for 30-45 minutes.

Cell lysis: Lysis step involved the use of a single cell lysis solution (CLS) comprising, 50 mM Tris base, 50 mM EDTA, lysozyme [2.5 mg/ml], pH 8.0) (Fisher-Scientific) plus an increased concentration of (PK) (Fisher-Scientific) (1.5 mg/ml) present. Plugs were lysed in 2 mL of CLS and placed in a shaking water bath at 37^{o}C for one hour.

Washing of the agarose plugs: The agarose plugs were washed 3 times; the first wash was with 5 mL of pre-heated sterile distilled water, and the other two washes were with 5 mL of pre-heated 0.1X TE buffer. The 0.1X TE buffer was prepared from 1X TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). All washes

were maintained at 55^{o}C in a shaking water bath, with each wash lasting exactly 15 minutes. Cold TE buffer was placed after the last wash and stored at 4^{o}C until further processing.

Restriction and running of plugs: restriction and running conditions were the same for both methods. The plugs were restricted with 40 Units of *Xba*I enzyme (Promega) with overnight incubation at 37^{o}C. For the gel itself, 1.2% pulsed-field certified agarose (Bio-Rad) was prepared. The running buffer was prepared from 10X Tris-Boric acid-EDTA buffer (TBE) (0.89 M Tris, 0.89 M Boric acid, 25 mM EDTA, pH 8.3) (Fisher) to a final concentration of 0.5X TBE. In the enhanced method further optimization was done by the addition of 50 µM Thiourea (Sigma) into the running buffer (Corkill *et al.*, 2000). As for running conditions, the gel was run for 20 hours at 14°C using CHEF-DRII system (Bio-Rad). The initial pulse was set at 5 seconds and the final pulse was altered to 40 seconds with a voltage of 6 (V/cm^{3}). The gels were then visualized using gelRed dye (Biotium) (Durmaz *et al.*, 2009, Koort *et al.*, 2002, Gautom, 1997).

3.3.3 Validation of the enhanced PFGE method

The enhanced method was validated by assessing its ability to type, power of discrimination and reproducibility. Results obtained from both methods were analysed using TIFF images with BioNumerics software, version 4. Clustering was performed using the un-weighted pair group method, arithmetic averages with Dice coefficient, position tolerance and an optimization of 1%. The cutoff clonal

relationship was defined based on the relationship of PFGE banding patterns to a single genetic event. In PFGE analysis, the value of such genetic event (e.g. point mutation, insertion or deletion of DNA) corresponds to 85% similarity (Ejrnaes *et al.*, 2006, Tenover *et al.*, 1995). Strains were referred as similar if they share ≥ 85% similarity. Strains were considered to be identical if they share 100% similarity (Ejrnaes *et al.*, 2006).

3.4 Sequencing with plasmid and genetic environment studies

The genetic environments of $bla_{CTX\text{-}M}$ genes found in our strains were studied using Genome Walking PCR (GW-PCR) as well as simplex PCR. Moreover, plasmid studies were employed to confirm the location of $bla_{CTX\text{-}M}$ genes on specific plasmids. In addition, studies included the detection of the sizes and the incompatibility groups (Inc) of the plasmids carrying $bla_{CTX\text{-}M}$ genes. The transferability of the plasmids carrying $bla_{CTX\text{-}M}$ genes was detected by performing conjugation studies.

Isolate №	Hospital	Strain
15	M	*E. coli*
20	M	*K. pneumoniae*
37	M	*K. pneumoniae*
47	M	*E. coli*
57	K	*E. coli*
61	K	*E. coli*
74	ID	*E. coli*
75	ID	*E. coli*
80	ID	*K. pneumoniae*
82	ID	*K. pneumoniae*
86	ID	*E. coli*
88	A	*K. pneumoniae*
89	A	*K. pneumoniae*
90	A	*E. coli*
91	A	*E. coli*
92	A	*E. coli*
93	A	*E. coli*
94	A	*E. coli*
95	A	*E. coli*
96	A	*E. coli*
97	A	*E. coli*
98	A	*E. coli*
99	A	*E. coli*
100	IB	*E. coli*
102	IB	*E. coli*

Table 11: Strains selected for sequencing and plasmid and genetic environment studies. A refers to the Al-Amiri hospital, IB the refers to the Ibn-Sina hospital, ID refers to the Infectious Diseases hospital, KOC refers to the Kuwait Oil Company hospital, and M refers to the Maternity hospital.

3.4.1 Selection of strains for the detection of the most prevalent CTX-M family member

PFGE was done to detect the clonal relatedness among the collected isolates. PFGE with MICs determination were supposed to help in selecting strains for further identification of the most prevalent CTX-M family member. Hypothetically, a representative strain from each clone with MDR pattern was to be amplified and sequenced. Unfortunately, no clonal relatedness was found among the strains except for 22 isolates. Therefore, strains were chosen for sequencing based on their MDR solely and disregarding their clonal pattern. In total, twenty two strains from all hospitals were used for further amplification. Upon sequencing, one CTX-M member of CTX-M-1 family was found to be most prevalent. Interestingly, derivatives of that member were reported and one parent enzyme. For further confirmation of the most prevalent CTX-M member, ten more strains were chosen for sequencing. Of the thirty-two isolates, twenty-one showed MDR pattern in reference to Zhanel (2008) criteria. The selection of more strains confirmed the commonest CTX-M member prevalent in all of the hospitals mentioned in this study. The selected strains are summarized in Table 12.

Isolate №	Hospital	Strain
4	M	*E. coli*
15	M	*E. coli*
17	M	*E. coli*
20	M	*K. pneumoniae*
37	M	*K. pneumoniae*
47	M	*E. coli*
55	K	*E. coli*
57	K	*E. coli*
60	K	*E. coli*
61	K	*E. coli*
74	ID	*E. coli*
75	ID	*E. coli*
80	ID	*K. pneumoniae*
82	ID	*K. pneumoniae*
86	ID	*E. coli*
87	ID	*E. coli*
88	A	*K. pneumoniae*
89	A	*K. pneumoniae*
90	A	*E. coli*
91	A	*E. coli*
92	A	*E. coli*
93	A	*E. coli*
94	A	*E. coli*
95	A	*E. coli*
96	A	*E. coli*
97	A	*E. coli*
98	A	*E. coli*
99	A	*E. coli*
100	IB	*E. coli*
101	IB	*E. coli*
102	IB	*E. coli*
105	IB	*E. coli*

Table 12: Strains selected for Identification of CTX-M-I group member. A refers to the Al-Amiri hospital, IB the refers to the Ibn-Sina hospital, ID refers to the Infectious Diseases hospital, KOC refers to the Kuwait Oil Company hospital, and M refers to the Maternity hospital. The strains highlighted in light yellow colour are the strains selected randomly.

For the identification of CTX-M-1 group members PCR amplification and sequencing was done with F 5'-CTTCCAGAATAAGGAATC-3' and R 5'-CCGTTTCCGCTATTACAA-3' at 52°C (Dutour *et al.*, 2002). All strains with a product size of 903 bp were sequenced. For sequencing, PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and sequenced using ABI3730 capillary sequencer (Applied Biosystems, Warrington, UK). The analysis of DNA sequencing results was done using BioEdit, Multalin, EXPASY, Basic Local Alignment Search Tool (BLAST), and European Bioinformatics Institute (EMBL) search engines. The resulting sequencing were compared to previously published CTX-M-15 gene sequences in an online website (http://www.lahey.org/studies/webt.stm) and (http://www.ncbi.nih.gov/BLAST) last access date (15th August 2012). The protein sequences were assembled using the EXPASY (expert analysis protein system) translate website (http:www.expasy.ch/tools/dna.html) last accessed (15th August 2012). Aligning the sequencing results and the protein sequences was done by the Multialin web site and EMBL-EBI online websites: (http://www.toulouse.inra.fr/multalin.html), and (http://www.ebi.ac.uk/) respectively last accessed (15th August 2012).

3.4.2 Selection of strains for plasmid and genetic environment studies

Out of the thirty-two strains selected for sequencing, twenty-five were chosen for the comparison of the genetic platforms and the description of possible common insertion sequences or other common transpositional elements. Also, the same twenty-five strains were used for further plasmid studies. Plasmid studies included the detection of the plasmid sizes carrying the *bla*$_{CTX-M}$ genes detected and the incompatibility grouping of the same plasmids. A list of the twenty-five strains used for this part of the study is summarized in Table 11.

3.4.3 Genome Walking and Simplex PCR for genetic environment studies

For the characterization of the genetic organization of the reported *bla*$_{CTX-M}$ genes found in this study, GW-PCR and simplex PCR were employed. Genome Walking PCR involved the use of specific nested primers for amplification and specific primers for direct sequencing as described (Pilhofer *et al.*, 2007, Cullik *et al.*, 2010). The primers sequences, annealing temperatures and PCR conditions used in GW-PCR are listed in Table 13 and Table 14. Simplex PCR was only performed for the characterization of the downstream region of *bla*$_{CTX-M-1}$ genes. Unfortunately, with GW-PCR there were no results for the detection of the downstream genetic context. Therefore, simplex PCR was done to detect the downstream area of the reported CTX-M genes

in this study. The primers used for the detection of the downstream regions in the simplex PCR are 1166Fo 5'-GCGATCCGCGTGATACCACT-3' and 1671Re 5'-CGTGGCTGCCGATGACTATG-3' (Sonnevend *et al.*, 2006). PCR products were analysed, purified, and sequenced as described in sections 3.1.

Primer Name	Primer Sequence	Round	PCR conditions	Reference
TSP2_X1_ +203	5'-CGCTCATCAGCACGATAAAG-3'	1	94°C – Denaturation – 4 minutes 94°C – 30 seconds 63°C – 30 seconds 72°C – 3 minutes } 30 cyles	(Cullik *et al.*, 2010, Pilhofer *et al.*, 2007)
		2	94°C – 30 seconds 40°C – 30 seconds 72°C – 3 minutes } 1 cycle	
		3	94°C – 30 seconds 63°C – 30 seconds 72°C – 3 minutes } 30 cycle 72°C – Final elongation – 10 minutes	
TSP3_X1_ +83	5'- GCATACAGCGGCACACTTC-3'	Direct sequencing		

Table 13: Primers sequences, annealing temperature and PCR conditions for GW-PCR for the detection of the up-stream environment of *bla*$_{CTX-M-1}$ group.

Primer Name	Primer Sequence	Round	PCR conditions	Reference
TSP7_X1_+821	5′- AGCCGTCGCGATGTATTAG-3′	1	94°C – Denaturation – 4 minutes 94°C – 30 seconds 63°C – 30 seconds cyles } 30 72°C – 3 minutes	(Cullik *et al.*, 2010, Pilhofer *et al.*, 2007)
		2	94°C – 30 seconds 40°C – 30 seconds cycle } 1 72°C – 3 minutes	
		3	94°C – 30 seconds 63°C – 30 seconds cycle } 30 72°C – 3 minutes 72°C – Final elongation – 10 minutes	
L7a_X1cons_+815	5′- GGCAGAAAGCCGTCGCGATGTATTAG-3′		Direct sequencing	

Table 14: Primers sequences, annealing temperature and PCR conditions for GW-PCR for the detection of the down-stream environment of *bla*$_{CTX-M-1}$ group.

3.4.4 Plasmid sizing and grouping

The sizing of plasmids was done using the prepared PFGE plugs containing genomic DNA. The method involved the use of nuclease S1 (Promega) to convert the circular plasmid into a linear form that allows a direct association between the distance that the DNA migrated in the running gels with its size. This protocol was described previously by Barton (1995). Ten units of nuclease S1 were used for each plug with the addition of 90 µL sterile distilled water and 10 µL nuclease S1 buffer (Promega). The plugs were incubated for 45 minutes at 37^{o}C and, to stop the enzymatic reaction, the plugs were washed with sterile distilled water. Running conditions and visualization of the gels were the same as those described in section 3.1 and 3.3.1.

For isolates shown to carry more than one plasmid, DNA gel extraction for each band was made using QIAquick gel extraction kit (Qiagen). Then, PCR amplification was done for each plasmid, using CTX-M primers; F 5'-SCSATGTGCAGYACCAGTAA-3' and R 5'-CCGCRATATGRTTGGTGGTG-3' (Saladin *et al.*, 2002). Further sequencing was done to confirm the presence of *bla*$_{CTX-M}$ member as described above in section 3.1. Then, PCR-based plasmid replicon typing was made as described previously (Carattoli *et al.*, 2005, Garcia-Fernandez *et al.*, 2009). The list of primers used for plasmid replicon typing is seen in Table 15. PCR reaction mix and gel visualization were done as described above in section 3.1 and 3.3.1.

Primer	Sequence	Product size	Annealing temperature	Reference
Inc R	5′-TCGCTTCATTCCTGCTTC AGC -3′ 5′-GTGTGCTGTGGTTATGCCTCA -3′	251 bp	52^{o}C	(Garcia-Fernandez *et al.*, 2009)
Inc N	5′-GTCTAACGAGCTTACCGAAG-3′ 5′-GTTTCAACTCTGCCAAGTTC-3′	559 bp	60^{o}C	(Carattoli *et al.*, 2005)
Inc FIA	5′-CCATGCTGGTTCTAGAGAAGGTG-3′ 5′-GTATATCCTTACTGGCTTCCGCAG-3′	462 bp	60^{o}C	(Carattoli *et al.*, 2005)
Inc FIB	5′-GGAGTTCTGACACACGATTTTCTG-3′ 5′-CTCCCGTCGCTTCAGGGCATT-3′	702 bp	60^{o}C	(Carattoli *et al.*, 2005)
Inc FII	5′-CTGTCGTAAGCTGATGGC-3′ 5′-CTCTGCCACAAACTTCAGC-3′	270 bp	52^{o}C	(Carattoli *et al.*, 2005)
Inc I1	5′-CGAAAGCCGGACGGCAGAA-3′ 5′-TCGTCGTTCCGCCAAGTTCGT-3′	139 bp	60^{o}C	(Carattoli *et al.*, 2005)
Inc L/M	5′-GGATGAAAACTATCAGCATCTGAAG-3′ 5′-CTGCAGGGGCGATTCTTTAGG-3′	785 bp	60^{o}C	(Carattoli *et al.*, 2005)

Table 15: Plasmid replicon typing primers with their annealing temperatures, product sizes, and references.

3.4.5 Conjugation studies

The transferability of the plasmids carrying *bla*$_{CTX-M}$ genes was carried out by broth mating. Strains carrying different *bla*$_{CTX-M}$ (15, 86, 89, 97 and 102) were used for conjugation studies. Our strains were used as donors and *E. coli* J62.2 (Rif^{R}) as recipient at a ratio of 1:10. Cultures of both donors and recipient were grown in 4.5 mL sterile nutrient broth and incubated overnight at 37^{o}C. Then, 100 µL of the donor's strain culture was added to 1 mL of the recipient strain. Transconjugants were selected on MacConkey agar supplemented with cefotaxime (32 mg/L) and rifampicin (50 mg/L) (Amyes and Gould, 1984). Controls were also prepared for donor and recipient strains by the inoculation of both onto other MacConkey plates with the same antibiotics. Confirmation of the success of the conjugation processes was done by PCR amplification mentioned in section 3.1. All of the agar and broth used in this experiment are from Oxoid Ltd (Basingstoke, UK). Cefotaxime and rifampicin used were from Sigma.

Moreover, the auto-transferability of the plasmids carrying *bla*$_{CTX-M}$ genes and which were shown to be Incompatibility group IncFII was studied. The technique was followed as described previously (Amyes and Gould, 1984). The method involved the use of 10^4 dilution of strains 91, 94, and 99 were cub-cultured on a series IST agar plates (Oxoid) containing cefotaxime (Sigma) in 10-fold concentrations. Then, 0.1 mL of an overnight culture broth of IST (Oxoid) of the same strains was mixed

with 4.5 mL a recipient strain (*E. coli* J62.2) of IST broth (Oxoid). The mixture was incubated for 24 hours at 37°C. Then, the recipients were selected on agar plates of IST with (32 mg/L cefotaxime) and (50 mg/L rifampicin).

3.5 Examination of the structural models of CTX-Ms found

Structural models of $bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}15}$, $bla_{CTX\text{-}M\text{-}55}$, and $bla_{CTX\text{-}M\text{-}117}$ were created with online protein homology/analogy recognition engine (phyre) (http:www.sbg.bio.ic.ac.uk/~phyre/) last access date (15th August 2012). The crystal structure for $bla_{CTX\text{-}M\text{-}9}$ published by Nichols *et al* (2012) was used as a backbone (Nichols *et al.*, 2012). Although, CTX-M-9 belongs to different CTX-M group than our reported CTX-Ms members, it had to be used as a backbone for our structure modelling examination because of the lack of published crystal structures of any member of CTX-M-1 group. Viewing of the structures was maintained using Swiss-Pdb Viewer DeepView version 4.0.4 (http://spdbv.vital-it.ch/) last access date (15th August 2012). Comparison of the structures was done to view amino-acid differences and how they affect the active site of the enzyme.

4 Results

4.1 Determination of MICs

Following the published BSAC breakpoints for the antibiotics used (cefepime, cefotaxime, cefoxitin, ceftazidime, ciprofloxacin, gentamicin, ertapenem, imipenem, and meropenem) (Andrews, 2012), the antibiotic profile for all of the isolates was determined as seen in Table 16. All of the *E. coli* (84) and *K. pneumoniae* (23) strains were resistant to cefotaxime and ceftazidime (100%) (Table 17and Table 18). Moreover, more than half of the *E. coli* strains (83.3%) were resistant to cefepime. Similarly, more than of half of the *K. pneumoniae* (86.95%) were resistant to cefepime. Also, more than half of the *E. coli* strains (70.23%) were resistant to ciprofloxacin. Likewise, more than half of the collected *K. pneumoniae* strains (73.91%) were resistant to ciprofloxacin. On the other hand, the resistant profiles of gentamicin of the collected *K. pneumoniae* isolates (73.91%) were higher than those for *E. coli* (57.14%) (Table 17 and Table 18). All of the collected isolate were sensitive to ertapenem, imipenem, and meropenem (Table 16).

Using the MDR definition set by Zhanel *et al* (2008), nineteen of the collected *E. coli* (22.61%) and 8 of the *K. pneumoniae* isolates (34.78%) had MDR patterns. The distribution of MICs at which 50% and 90% of the isolates were inhibited (MIC_{50} and MIC_{90}, respectively) for cefepime, cefotaxime, cefoxitin, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem, and ertapenem are given in Table 19. The

MIC_{50} and the MIC_{90} of both strains help us to understand the proportion of resistance in the collected isolates. Overall, the MIC_{50} and MIC_{90} (in mg/L) for *E. coli* were as follows: cefepime, 16 and 64; cefotaxime, 128 and 256; cefoxitin, 8 and 64; ceftazidime, 32 and >32; ciprofloxacin, 64 and >256; gentamicin, 32 and 32; imipenem, 0.03 and 0.12; meropenem, 0.03 and 0.25; ertapenem, 0.5 and 0.5. In addition, the MIC_{50} and MIC_{90} (in mg/L) for *K. pneumoniae* were as follows: cefepime, 16 and 32; cefotaxime, 128 and >256; cefoxitin, 8 and 16; ceftazidime, 32 and >32; ciprofloxacin, 8 and >256; gentamicin, 32 and 128; imipenem, 0.015 and 0.5; meropenem, 0.06 and 0.25; ertapenem, 0.5 and 0.5. The MIC_{50} of cephalosporins to *E. coli* were the same as those to *K. pneumoniae*. However, the MIC_{90} of cephalosporins (except cefepime) in *E. coli* were lower by 1-3 folds than those to *K. pneumoniae*. Interestingly, the MIC_{90} of cefepime in *E. coli* was one fold higher than the MIC_{90} of the same antimicrobial agent to *K. pneumoniae*.

Regarding *E. coli*, major disparities were found between the MIC_{50} of cephalosporins (cefepime, cefotaxime, cefoxitin, and ceftazidime). Whereas, no major increase in the dilutions of MIC_{50} of cephalosporins to *K. pneumoniae* was seen. Similarly, the MIC_{90} of cephalosporins (except cefoxitin) to both strains (*E. coli* and *K. pneumoniae*) had no major differences in dilutions; ranging from one to two folds. The MIC_{90} of cefoxitin to *E. coli* was three dilution steps higher than the MIC_{50} of the same drugs to the same strain.

In terms of the MIC_{50} of ciprofloxacin to both strains, the MIC_{50} of ciprofloxacin to *E. coli* was three dilution steps higher than that found in *K. pneumoniae*. On the other hand, the MIC_{90} of ciprofloxacin to both strains was the same.

In case of gentamicin, the MIC_{90} of this antimicrobial agent to *K. pneumoniae* was two folds higher than that to *E. coli*. While the MIC_{50} of both strains was the same.

When comparing the MIC_{50} and the MIC_{90} of carbapenems to *E. coli* and *K. pneumoniae*, no major difference in dilutions was observed. Notably, the MIC_{90} of imipenem to *K. pneumoniae* was five dilutions steps higher than the MIC_{50} of the same antimicrobial drug to *K. pneumoniae* strains. In addition, the MIC_{50} of meropenem to *E. coli* was three folds higher than the MIC_{90} of meropenem to the same strain.

Isolate №	Strain	MIC (mg/L)								
		Cefepime	Cefotaxime	Cefoxitin	Ceftazidime	Ciprofloxacin	Gentamicin	Ertapenem	Imipenem	Meropenem
1	*E. coli*	8	32	16	32	32	0.25	0.03	0.015	0.25
2	*E. coli*	16	8	4	32	0.03	32	0.03	0.008	0.25
3	*E. coli*	32	128	16	32	>256	32	0.12	0.06	0.25
4	*E. coli*	1	4	4	32	0.5	0.5	0.03	0.015	0.25
5	*K. pneumoniae*	0.5	4	8	32	0.5	1	0.12	0.03	0.25
6	*E. coli*	16	64	8	32	64	16	0.06	0.008	0.25
7	*K. pneumoniae*	32	128	2	32	0.12	64	0.06	0.03	0.25
8	*E. coli*	16	64	16	32	64	32	0.06	0.03	0.25
9	*K. pneumoniae*	8	32	16	32	64	8	0.06	0.03	0.25
10	*E. coli*	8	64	4	32	0.25	32	0.12	0.06	0.25
11	*E. coli*	16	8	8	32	0.06	0.5	0.03	0.008	0.25
12	*E. coli*	8	8	8	32	128	64	0.03	0.03	0.25
13	*E. coli*	8	64	16	32	0.12	64	0.06	0.008	0.25
14	*E. coli*	32	128	256	32	0.12	256	0.06	0.015	0.25
15	*E. coli*	8	128	16	32	>256	64	0.03	0.015	0.25
16	*K. pneumoniae*	16	128	4	32	4	64	0.06	0.015	0.25
17	*E. coli*	32	128	16	32	128	32	0.03	0.015	0.25
18	*K. pneumoniae*	16	128	8	32	64	64	0.03	0.008	0.25
19	*K. pneumoniae*	8	64	8	32	0.25	32	0.03	0.012	0.25
20	*K. pneumoniae*	8	32	8	32	>256	32	0.03	0.015	0.25
21	*E. coli*	16	64	8	32	0.03	128	0.008	0.008	0.25
22	*E. coli*	4	128	8	32	0.25	0.5	0.12	0.06	0.25
23	*E. coli*	8	16	8	32	128	0.5	0.03	0.015	0.25
24	*E. coli*	4	128	16	32	0.25	1	0.06	0.008	0.25
25	*E. coli*	8	16	8	32	0.25	64	0.06	0.06	0.25
26	*E. coli*	8	32	8	32	>256	1	0.06	0.03	0.25
27	*E. coli*	8	64	4	32	0.25	1	0.5	0.25	0.12

Isolate №	Strain	MIC (mg/L)								
		Cefepime	Cefotaxime	Cefoxitin	Ceftazidime	Ciprofloxacin	Gentamicin	Ertapenem	Imipenem	Meropenem
28	*K. pneumoniae*	32	>256	16	32	256	32	0.5	0.25	0.12
29	*K. pneumoniae*	1	4	4	32	4	128	0.5	0.25	0.12
30	*E. coli*	8	128	4	32	0.25	32	0.5	0.12	0.12
31	*K. pneumoniae*	16	256	8	32	1	1	0.5	0.03	0.12
32	*K. pneumoniae*	32	256	8	32	1	0.25	0.5	0.008	0.12
33	*E. coli*	16	128	8	32	32	0.25	0.5	0.015	0.12
34	*E. coli*	64	>256	8	32	1	1	0.03	0.008	0.03
35	*E. coli*	16	64	4	32	16	1	0.05	0.03	2
36	*E. coli*	16	256	4	32	0.015	64	0.25	0.03	2
37	*K. pneumoniae*	32	>256	4	32	>256	64	0.5	0.008	0.03
38	*K. pneumoniae*	16	>256	2	32	4	64	0.5	0.25	0.03
39	*E. coli*	16	256	8	32	8	64	0.5	0.12	0.03
40	*E. coli*	16	64	4	32	256	1	0.12	0.03	0.03
41	*K. pneumoniae*	4	32	4	32	32	0.5	0.5	0.12	2
42	*E. coli*	4	64	4	32	32	0.25	0.03	0.008	2
43	*K. pneumoniae*	32	>256	8	32	256	64	0.5	0.03	0.03
44	*K. pneumoniae*	16	>256	2	32	8	64	0.5	0.008	0.06
45	*K. pneumoniae*	8	64	4	32	16	64	0.5	0.008	0.03
46	*K. pneumoniae*	32	>256	4	32	0.25	4	0.5	0.008	0.03
47	*E. coli*	64	>256	128	32	256	64	0.03	0.03	0.06
48	*E. coli*	32	256	8	32	2	32	0.5	0.03	0.03
49	*E. coli*	16	256	4	32	0.5	32	0.03	0.03	0.06
50	*E. coli*	16	>256	8	32	0.5	1	0.5	0.03	0.03
51	*E. coli*	32	256	16	32	16	64	0.5	0.03	0.06
52	*E. coli*	32	256	64	32	>256	1	0.12	0.06	0.03
53	*E. coli*	32	256	64	32	>256	1	0.06	0.008	0.015
54	*E. coli*	32	64	256	32	4	2	0.5	0.25	0.015
55	*E. coli*	8	64	8	32	32	16	0.12	0.06	0.015

Isolate №	Strain	MIC (mg/L)								
		Cefepime	Cefotaxime	Cefoxitin	Ceftazidime	Ciprofloxacin	Gentamicin	Ertapenem	Imipenem	Meropenem
56	*E. coli*	32	128	16	32	128	2	0.12	0.06	0.015
57	*E. coli*	16	128	32	32	>256	16	0.5	0.12	0.015
58	*E. coli*	16	64	8	32	128	32	0.12	0.05	0.015
59	*E. coli*	2	128	8	32	128	64	0.5	0.03	0.015
60	*E. coli*	2	128	2	32	128	256	0.06	0.06	0.015
61	*E. coli*	64	256	64	32	256	64	0.06	0.008	0.03
62	*E. coli*	8	64	8	32	64	32	0.06	0.06	0.015
63	*E. coli*	32	256	8	32	64	32	0.25	0.06	0.015
64	*E. coli*	16	64	8	32	0.5	1	0.06	0.008	0.015
65	*E. coli*	32	256	16	32	128	1	0.06	0.015	0.015
66	*E. coli*	32	256	64	32	>256	64	0.06	0.015	0.015
67	*E. coli*	4	32	8	32	64	64	0.06	0.015	0.015
68	*E. coli*	16	64	8	32	64	0.5	0.06	0.015	0.015
69	*E. coli*	16	128	16	32	128	16	0.06	0.008	0.015
70	*E. coli*	32	256	16	32	256	32	0.5	0.008	0.008
71	*E. coli*	32	256	32	32	128	0.25	0.5	0.008	0.008
72	*E. coli*	8	64	4	32	128	0.25	0.5	0.12	0.008
73	*E. coli*	4	32	4	32	0.5	32	0.5	0.12	0.008
74	*E. coli*	32	128	32	32	256	64	0.5	0.008	0.008
75	*E. coli*	16	64	8	32	128	64	0.5	0.015	0.008
76	*E. coli*	16	256	16	32	256	32	0.5	0.015	0.008
77	*E. coli*	32	64	8	32	256	32	0.5	0.015	0.008
78	*E. coli*	16	256	8	32	256	0.25	0.5	0.015	0.008
79	*E. coli*	64	64	8	32	256	64	0.5	0.015	0.008
80	*K. pneumoniae*	32	256	16	32	256	32	0.5	0.008	0.008
81	*E. coli*	32	256	32	32	128	0.25	0.5	0.015	0.008
82	*K. pneumoniae*	32	>256	8	32	256	16	0.5	0.008	0.06
83	*E. coli*	4	128	16	32	128	16	0.5	0.008	0.008

Isolate №	Strain	MIC (mg/L)								
		Cefepime	Cefotaxime	Cefoxitin	Ceftazidime	Ciprofloxacin	Gentamicin	Ertapenem	Imipenem	Meropenem
84	*E. coli*	32	256	16	32	256	32	0.5	0.008	0.008
85	*K. pneumoniae*	32	256	32	32	>256	0.5	0.5	0.008	0.008
86	*E. coli*	16	128	8	32	256	32	0.5	0.06	0.008
87	*E. coli*	64	32	32	32	256	0.25	0.5	0.06	0.008
88	*K. pneumoniae*	16	128	16	32	4	128	0.5	0.12	0.06
89	*K. pneumoniae*	128	128	64	64	128	128	0.5	0.015	0.06
90	*E. coli*	8	128	128	32	0.12	128	0.5	0.12	0.06
91	*E. coli*	8	128	4	32	0.25	64	0.5	0.03	0.06
92	*E. coli*	0.5	8	8	32	0.12	64	0.5	0.008	0.06
93	*E. coli*	8	128	8	32	8	128	0.5	0.015	0.06
94	*E. coli*	128	128	64	32	8	128	0.5	0.12	0.06
95	*E. coli*	128	128	64	32	0.25	64	0.5	0.12	0.06
96	*E. coli*	64	128	64	32	0.12	64	0.5	0.12	0.06
97	*E. coli*	256	256	8	8	>256	32	0.5	0.008	0.03
98	*E. coli*	32	32	8	32	>256	4	0.5	0.015	0.06
99	*E. coli*	64	128	64	32	8	256	0.5	0.03	0.06
100	*E. coli*	64	256	32	32	256	32	0.5	0.03	0.008
101	*E. coli*	16	128	8	32	256	32	0.5	0.5	0.008
102	*E. coli*	32	256	8	8	128	32	0.5	0.03	0.008
103	*E. coli*	64	128	>256	32	256	0.25	0.5	0.12	0.008
104	*E. coli*	64	256	64	32	128	0.25	0.5	0.5	0.008
105	*E. coli*	64	128	64	32	128	8	0.5	0.008	0.008
106	*E. coli*	64	256	32	32	256	32	0.5	0.008	0.25
107	*E. coli*	32	128	8	32	32	32	0.5	0.12	0.008

Table 16: Minimum inhibitory concentration values (mg/L) with four classes of antibiotics. Light blue fields highlight resistance to antibiotic used, pink highlighted fields are isolates with an MDR pattern selected for further studies, and green highlighted fields are strains with no MDR pattern but selected for plasmid studies.

Antibiotic	Number of resistant isolates	% of resistance	Range of MIC (ml/L)
Cefepime	70	83.3	8—256
Cefotaxime	84	100	4—>256
Cefoxitin	39	46.42	16—>256
Ceftazidime	84	100	32
Ciprofloxacin	59	70.23	8—>256
Gentamicin	48	70.23	16—256
Imipenem	0	0	0.008—0.5
Meropenem	0	0	0.008—0.25
Ertapenem	0	0	0.008—2

Table 17: Proportion of resistance in *E. coli* isolates.

Antibiotic	Number of resistant isolates	% of resistance	Range of MIC (ml/L)
Cefepime	20	86.95	8—32
Cefotaxime	23	100	4—>256
Cefoxitin	9	39.13	4—64
Ceftazidime	23	100	32
Ciprofloxacin	17	73.91	4—>256
Gentamicin	17	73.91	8—128
Imipenem	0	0	0.008—0.25
Meropenem	0	0	0.008—2
Ertapenem	0	0	0.03—0.5

Table 18: Proportion of resistance in *K. pneumoniae* isolates.

Organism (№ of isolates)	Antimicrobial agent	MIC_{50}	MIC_{90}	Range of MIC (ml/L)
E. coli (84)	Cefepime	16	64	8—256
	Cefotaxime	128	256	4—>256
	Cefoxitin	8	64	16—256
	Ceftazidime	32	32	32
	Ciprofloxacin	64	>256	8—>256
	Gentamicin	32	32	16—256
	Imipenem	0.03	0.12	0.008—0.25
	Meropenem	0.03	0.25	0.12—0.25
	Ertapenem	0.5	0.5	0.008—0.25
K. pneumoniae (23)	Cefepime	16	32	8—32
	Cefotaxime	128	>256	4—>256
	Cefoxitin	8	16	4—64
	Ceftazidime	32	32	32
	Ciprofloxacin	8	>256	4—>256
	Gentamicin	32	128	8—128
	Imipenem	0.015	0.5	0.008—0.25
	Meropenem	0.06	0.25	0.008—2
	Ertapenem	0.5	0.5	0.03—0.5

Table 19: MIC_{50} and MIC_{90} of *E. coli* and *K. pneumoniae.*

4.2 Phenotypic and molecular identification of *bla*$_{CTX-M}$ genes

With the Vitek 2 system, all of the isolates were noted for ESBL-producer production. The DDD method with cefotaxime and ceftazidime confirmed the presence of CTX-M-production. For further conformation, PCR was used to detect CTX-M enzymes genotypically. Using the primers described by Saladin (2002), it was found that all of the isolates (107 isolates) encoded *bla*$_{CTX-M}$ genes. All of the isolates showed a product size of 504 bp when amplified. Some of the isolates producing CTX-M enzymes are seen in Figure 8. Further amplification with other primers (CTX-M-1 group primers) listed in Table 9 showed that all of the isolates in this study carry *bla*$_{CTX-M}$ genes belonging to CTX-M-1 group. This can be seen in Figure 9.

When using primers for CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 sub-groups, listed in Table 9, no bands were observed. This indicated that all of the isolates collected in this study encode CTX-M genes belonging to CTX-M-1 sub-group only. Also, I confirm the absence of *bla*$_{CTX-M}$ genes belonging to other CTX-M sub-families.

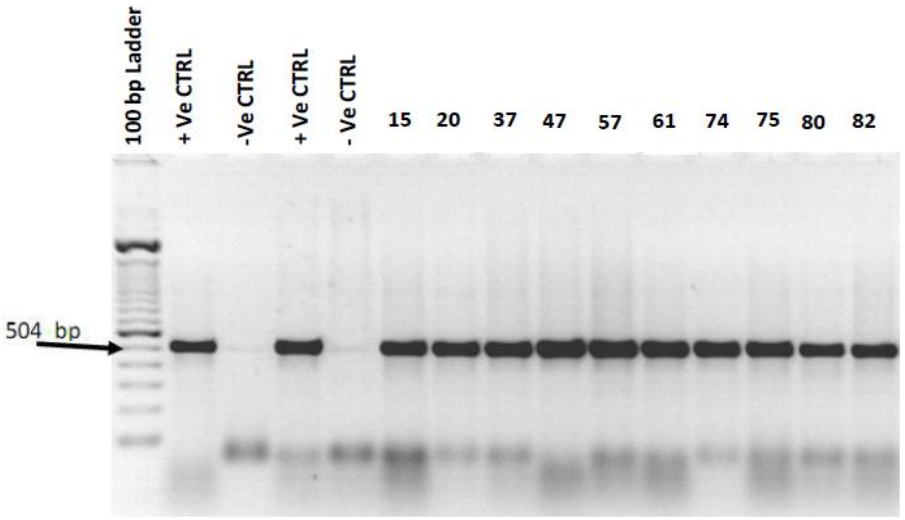


Figure 8: PCR products using CTX-M consensus primers (Saladin *et al.*, 2002). The expected product size is 504 bp. +ve CTRL indicates positive control, -ve CTRL indicates negative control,15, 20, 37, 47, 57, 61, 74, 75, 80, and 82 represent isolate numbers.

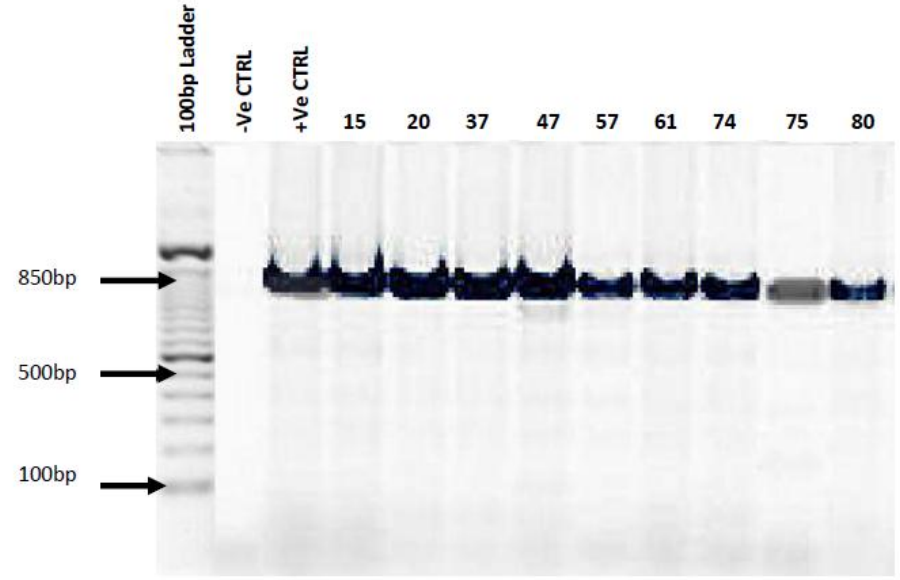


Figure 9: PCR products using CTX-M-1 group designed primers. The expected product size is 854bp. +ve CTRL indicates positive control, -ve CTRL indicates negative control, 15, 20, 37, 47, 57, 61, 74, 75, 80, and 82 represent isolate numbers.

4.3 Pulsed-field gel electrophoresis

4.3.1 Enhanced rapid method

Molecular fingerprinting was done to establish the clonality of isolates collected over a reasonably short period of time (2006-2010). Also, the use of PFGE was retained to help in the choice of a representative strain from each clone for further identification by sequencing the members of CTX-M-1 group and eventual determination of the most common CTX-M-1 group-producer. All of the collected isolates (except for 22) showed different PFGE patterns with less than 85% similarity and did not share clonal relatedness was found among them, as seen in Figure 10 and Figure 11. The remaining 22 isolates exhibited banding patterns that allowed grouping them into 12 distinct PFGE clusters as seen in Figure 10 and Figure 11. It is notable that the strains sharing ≥85% were from the same hospitals. Unfortunately, insufficient information during data collection was found and there was no evidence of genotypic relatedness in samples collected from patients from the same ward. Therefore, we cannot indicate the transmission of a bacterial pathogen from patients in the same ward and the only notification is that genotypic relatedness was observed from isolates collected from the same hospital.

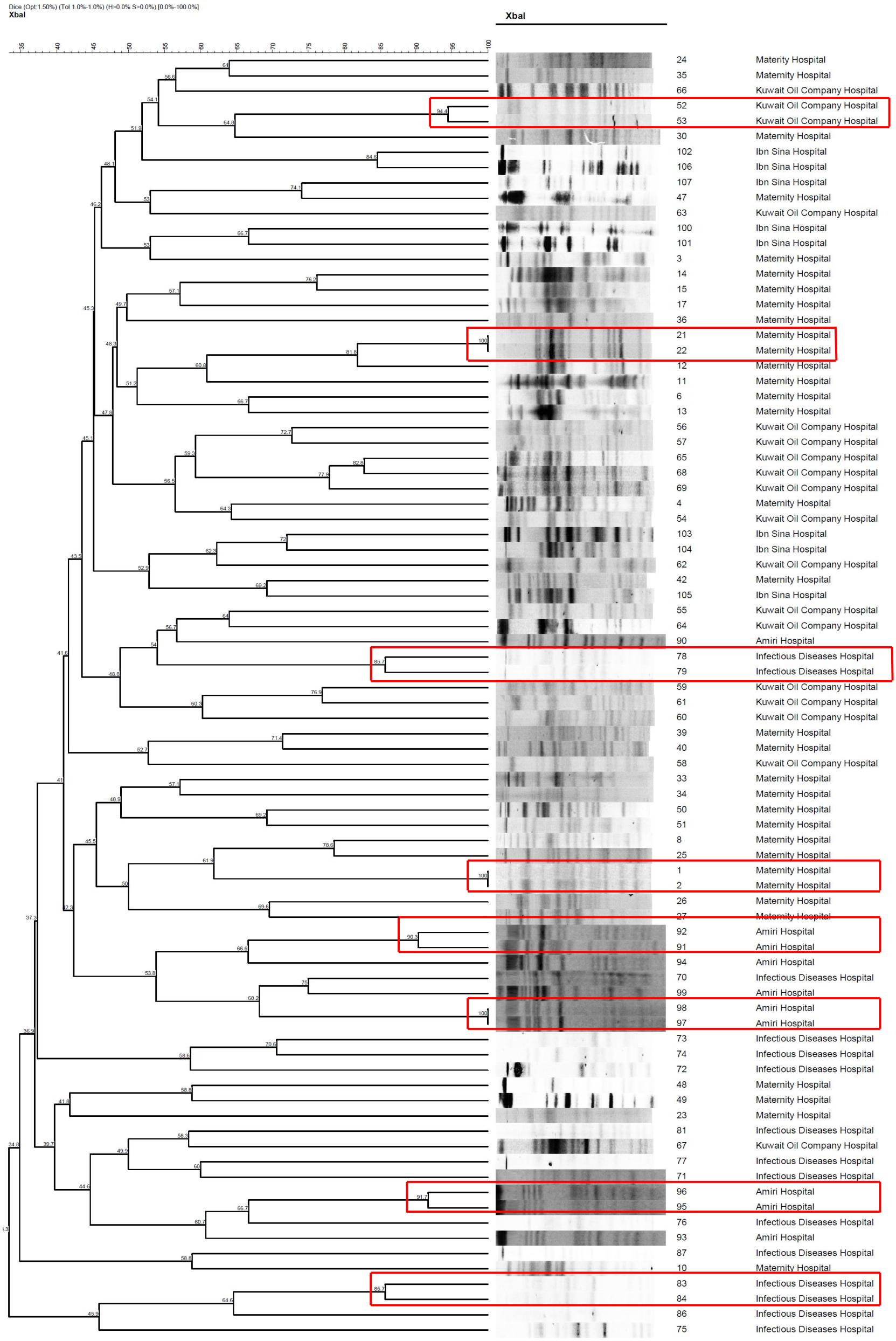


Figure 10: PFGE banding patterns with *E. coli* Strains. Strains highlighted with red colour share ≥85% similarity.

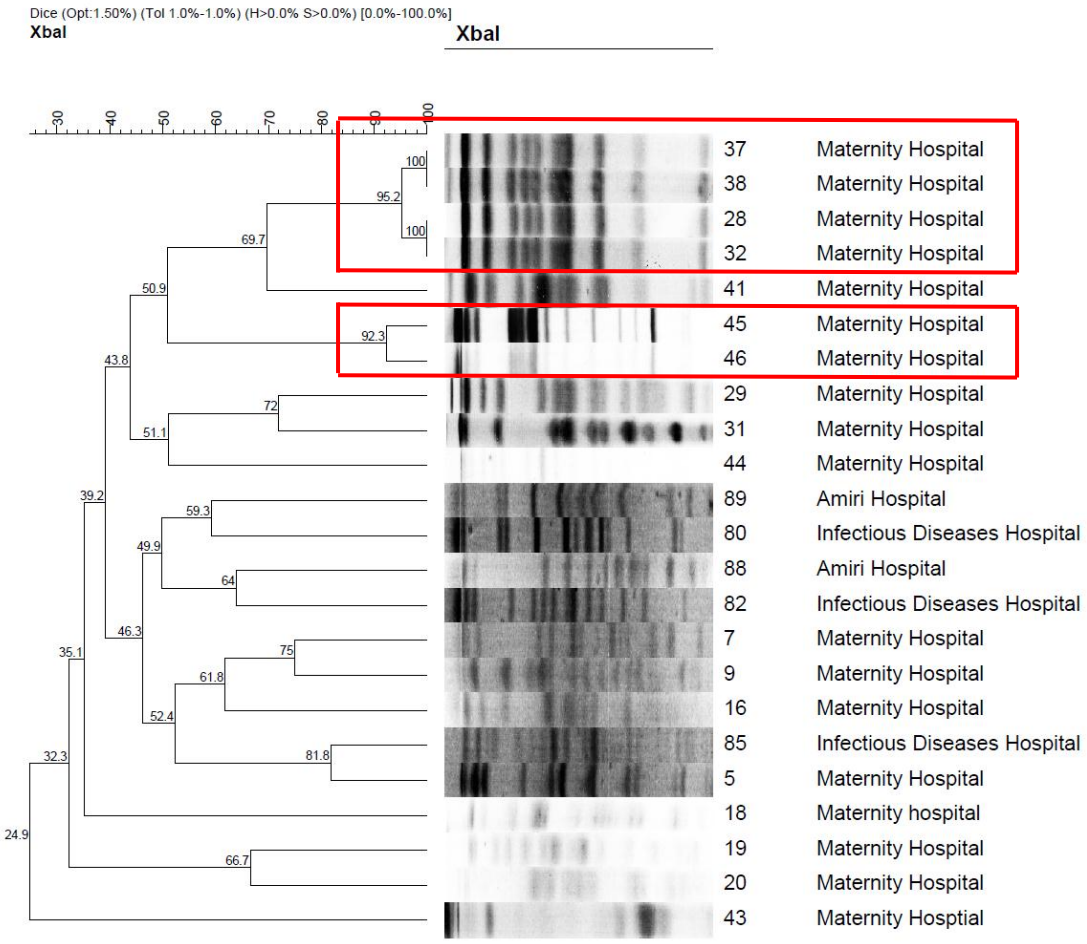


Figure 11: PFGE banding patterns with *K. pneumoniae* Strains. Strains highlighted with red colour share ≥85% similarity.

4.3.2 Prolonged PFGE method

The restriction banding patterns obtained by the prolonged PFGE protocol were analysed and compared with the results of the enhanced shortened protocol as seen in Figure 12, Figure 13, Figure 14, Figure 15, Figure 16, and Figure 17. In Figure 12, the similarity values of *E. coli* strains provided by the traditional PFGE method were the same as those when using the shortened technique as seen in Figure 13. In addition, the similarity values of *K. pneumoniae* strains that are similar and identical are the same using both techniques (Figure 14 and Figure 15). The similarity values of identical/similar *E. coli* and *K. pneumoniae* strains with both techniques are summarized in Table 22 and Table 21.

It was found that the similarity values obtained from both techniques were the same for the 30 unrelated *E. coli* strains selected for validation as seen in Figure 16 and Figure 17. Moreover, the similarity results for the 30 unrelated *E. coli* strains are listed in Table 22.

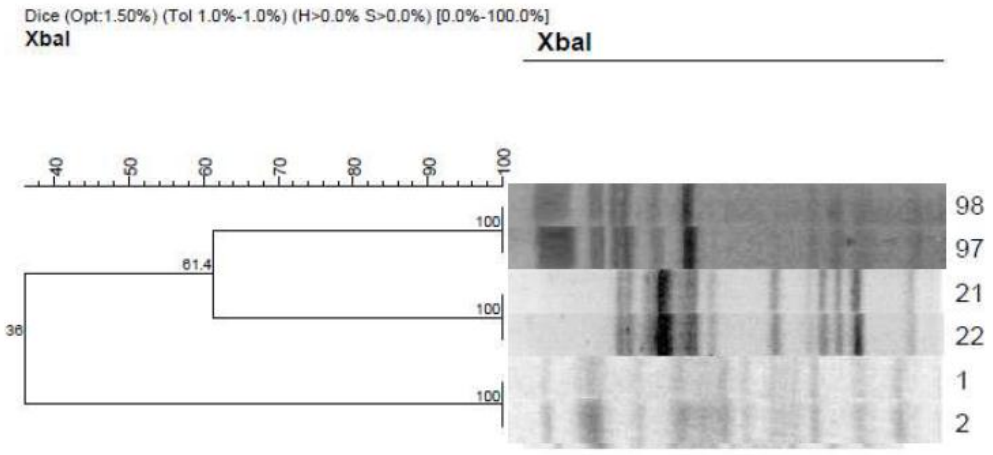


Figure 12: PFGE banding patterns obtained with six *E. coli* strains. DNA band profiles show 100% similarity for three groups of two strains.

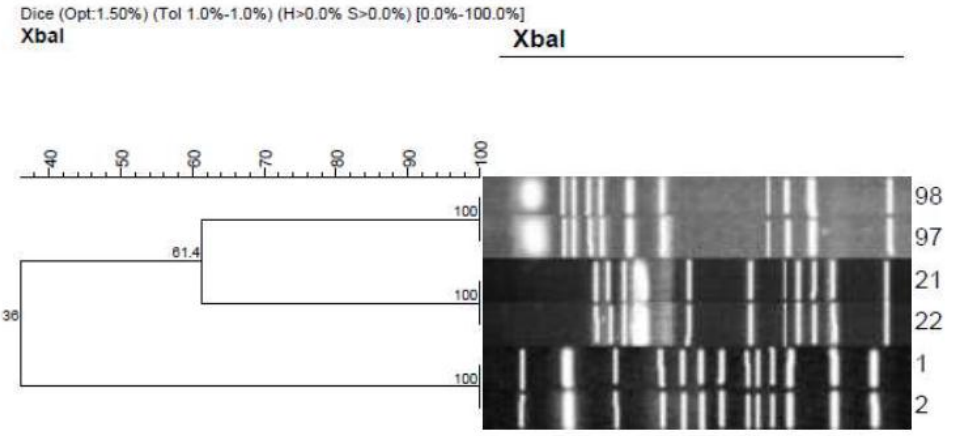


Figure 13: PFGE banding patterns obtained with six *E. coli* strains. PFGE fingerprinting patterns obtained with the enhanced Durmaz *et al.* method.

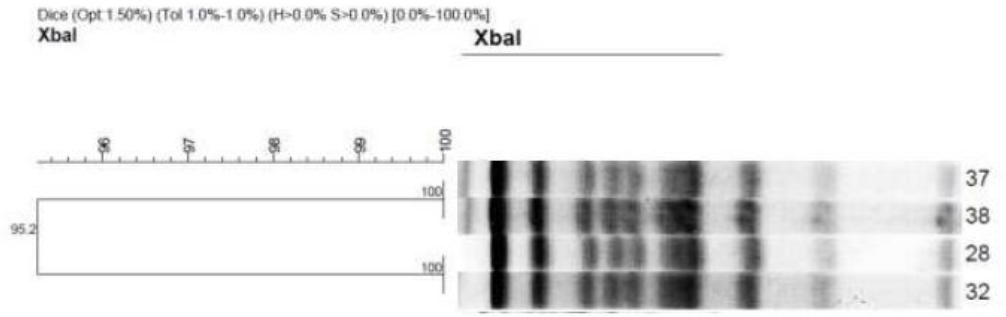


Figure 14: PFGE banding patterns obtained with four *K. pneumoniae* strains. DNA band profiles using the traditional PFGE protocol, showing 100% similarity for most of all of the strains.

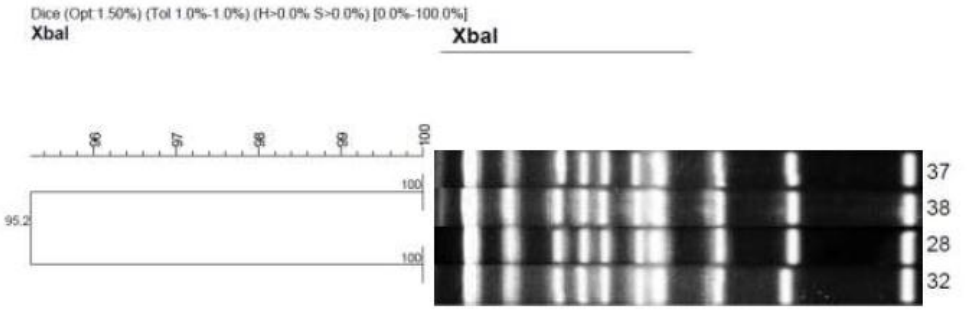


Figure 15: PFGE banding patterns obtained with four *K. pneumoniae* strains. PFGE fingerprinting patterns obtained with the enhanced Durmaz *et al.* method.

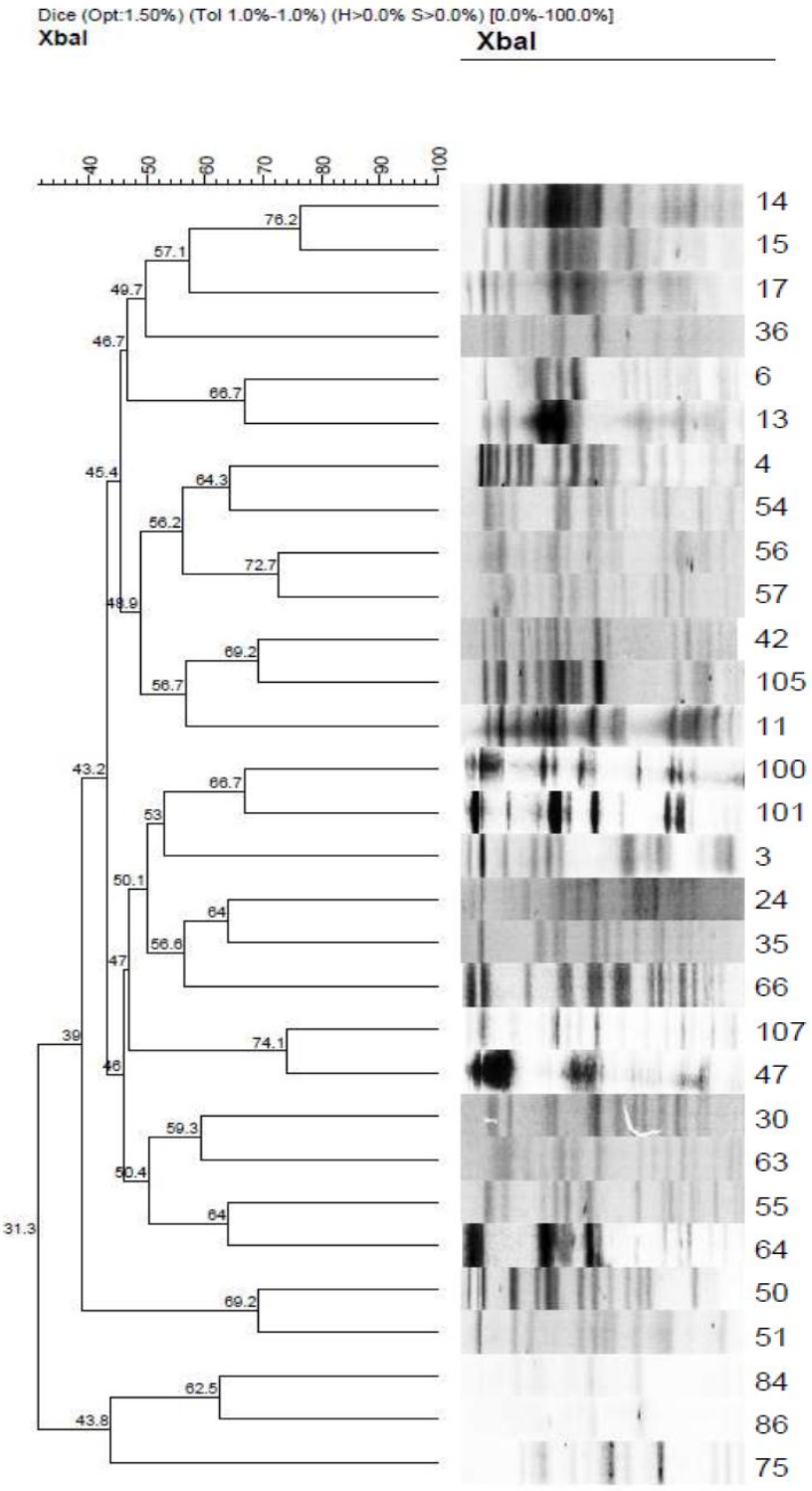


Figure 16: PFGE banding patterns obtained with 30 *E. coli* strains. DNA band profiles using the traditional PFGE protocol.

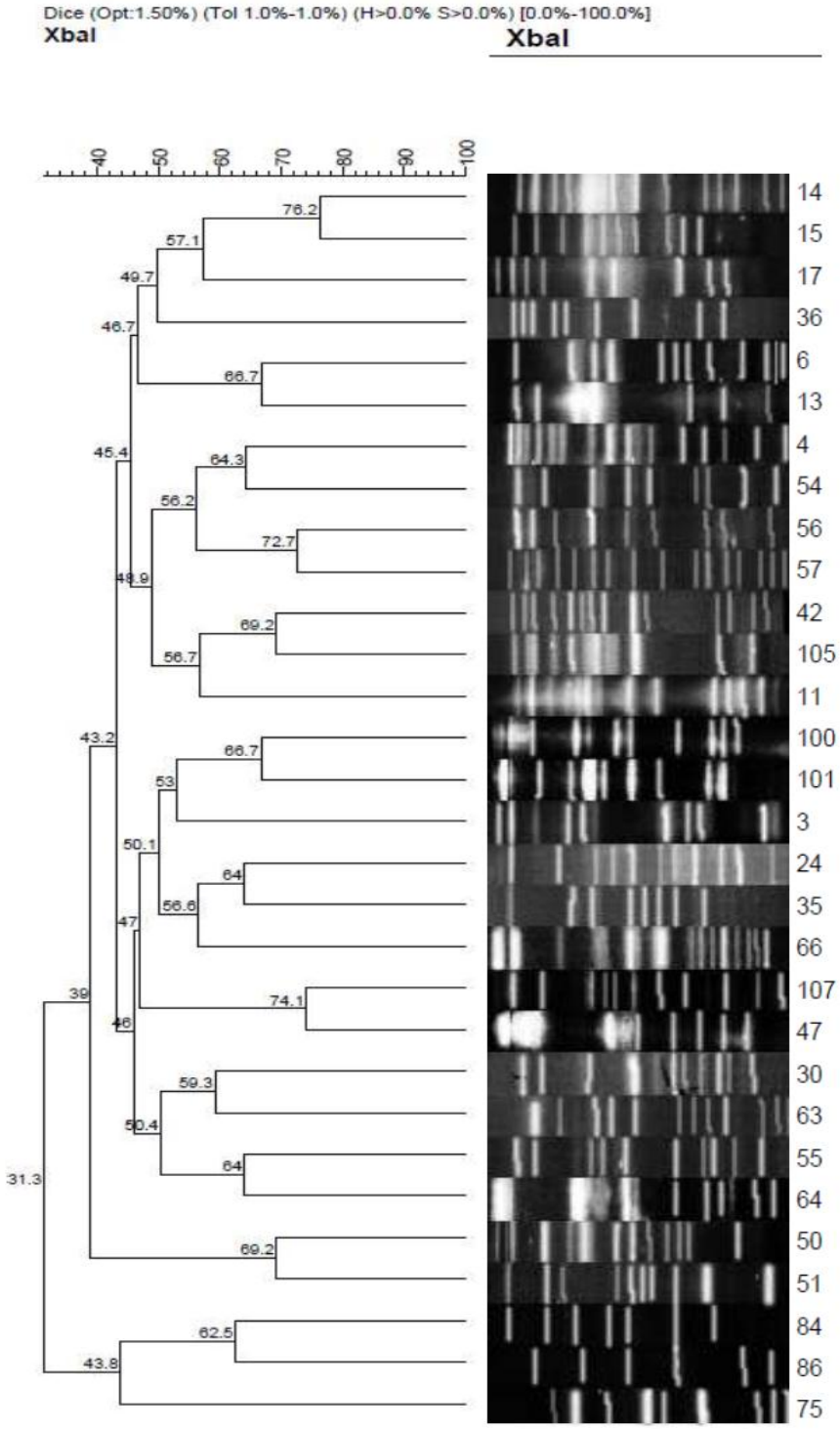


Figure 17: PFGE banding patterns obtained with 30 *E. coli* strains. PFGE fingerprinting patterns obtained with the enhanced Durmaz *et al.* method.

Strain Number	Percentage of similarity values using the enhanced shortened genotyping method
1-2	100
21-22	100
28-32	100
37-38	100
(28-32)-(37-38)	95.2
45-46	92.3
52-53	94.4
78-79	85.7
83-84	85.7
91-92	90.3
95-96	91.7
97-98	100

Table 20: Similarity values of strains ≥85% similarity.

Strain Number	Similarity values using the enhanced shortened genotyping method	Percentage of similarity values using the prolonged genotyping method
1-2	100%	100
21-22	100%	100
28-32	100%	100
37-38	100%	100
97-98	100%	100

Table 21: Similarity values of identical strains pulsed by the prolonged PFGE method and the enhanced rapid method and analysed by BioNumerics software, version 4.

Strain Number	Percentage of the similarity values using the prolonged genotyping method	Percentage of the similarity values using the enhanced shortened genotyping method
14-15	76.2	76.2
17- (14-15)	57.1	57.1
36- (14-15-17)	49.7	49.7
6-13	66.7	66.7
4-54	64.3	64.3
56-57	72.7	72.7
42-105	69.2	69.2
11 - (42-105)	56.7	56.7
100-101	66.7	66.7
3 - (100-101)	53	53
24-35	64	64
66 - (24-35)	56.6	56.6
47-107	74.1	74.1
30-63	59.3	59.3
55-64	64	64
50-51	69.2	69.2
84-56	62.5	62.5
75 - (84-86)	43.8	43.8

Table 22: Similarity values of epidemiologically unrelated *E. coli* strains pulsed by the prolonged PFGE method and the enhanced rapid method and analysed by BioNumerics software, version 4.

4.3.3 Validation of the enhanced PFGE method

The assessment of a typing method should fulfil the definition of the following terms; typeability, discriminatory power, reproducibility, and ease of performance and interpretation (Singh *et al.*, 2006). In terms of typeability, the newly described PFGE fingerprinting protocol showed typeability by yielding interpretable PFGE profiles. The lengthy fingerprinting procedure, to which it was compared, was also, of course, considered typeable.

Upon repetition of the enhanced PFGE procedure, the PFGE banding patterns provided were the same. Accordingly, I can define that the new technique as reproducible. This reproducibility was, of course, a defining feature of the lengthy PFGE procedure.

Mainly, the validation is assessed by the comparison of restriction endonuclease banding patterns of epidemiologically identical strains with a more traditional typing method. Therefore, I validated the shortened PFGE method by comparing the similarity values of the epidemiologically identical strains with both techniques (1 and 2, 21 and 22, 28 and 32, 37 and 38, 97 and 98). Strains examined for the validation of the protocol (1 and 2, 21 and 22, 28 and 32, 37 and 38, 97 and 98) were found to be identical in both procedures, showing 100% similarity as seen in Figure 12, Figure 13, Figure 14, and Figure 15. From the results seen in Table 20 and

Table 21, I can confirm the validity of the shortened genotyping method for typing *E. coli* and *K. pneumoniae*.

The discriminatory power of both techniques was assessed by the comparison of the similarity values of epidemiologic unrelated strains (3, 4, 6, 11, 13, 14, 15, 17, 24, 30, 35, 36, 42, 47, 50, 51, 54, 55, 56, 57, 63, 64, 66, 75, 84, 86, 100, 101, 105, and 107), using BioNumerics software, version 4. The similarity values of unrelated strains were exactly the same with both protocols as seen in Figure 16 and Figure 17. The similarity value results are documented in Table 22. Therefore, I can confirm the discriminatory power of the enhanced PFGE method in comparison with that of Miranda *et al* technique (1996).

In the traditional protocol for genotyping bacterial strains, a colony from a pure culture is inoculated in Brain Heart broth overnight. While in the followed method described herein, no further inoculation of a pure culture is required. This took out a day from the genotyping procedure. During the lysis steps, in the original PFGE method the cells were lysed for two days. On the other hand, our enhanced protocol involved lyses of cells only for one hour. This shortened PFGE method by 2 days. The concentration of PK was manipulated to reach 1.5 mg/ml to reduce the time required for lysing the cells (Alaidan *et al.*, 2009). Additionally, the lysis solution should be freshly prepared to obtain optimized action of proteinase K (Murray *et al.*, 1990). Washing of the plugs was modified in terms of time required for each wash. Each wash was done for 15 minutes rather than 30 minutes, due to

the use of a shaking water bath and pre-heated washing buffers which ensure complete wash of the plugs (Alaidan *et al.*, 2009). Lower concentration of TE buffer was used as 0.1X TE in the last two washes. The concentration of the washing buffer was diluted to 0.1X TE to reduce the effect of nucleolytic peracid derivatives of Tris salts formed, which in some cases interferes with the action of restriction enzymes used and in return affecting the banding pattern giving a washed out appearance upon running (Corkill *et al.*, 2000, Matushek *et al.*, 1996, Bannerman *et al.*, 1995). The use of thiourea in the running buffer was indicated to prevent the DNA degradation of some sensitive strains. The degradation of DNA remains ambiguous but is thought that thiourea acts as a neutralizer to nucleolytic peracid derivatives produced by Tris salts in TBE buffer (Corkill *et al.*, 2000, Koort *et al.*, 2002, Romling and Tummler, 2000, Bush and Jacoby, 2010). In some reports, instead of lowering the concentration of Tris salts, Tris buffers were replaced by the use of HEPES to prevent the reaction of Tris radicals (Romling and Tummler, 2000). The disadvantage of such replacement lies with the cost of HEPES. The main goal of this method was to provide a rapid and cost-effective protocol rather than using expensive materials.

The time required for genotyping strains with the traditional method was 6 days, while modifications made removed 4 days from the original procedure. This method was also optimized to be a cost-effective method. This typing system could be characterized by means of comparable reproducibility and discriminatory power. It could also be used in large laboratories where cost is an issue, as most of the

materials/buffers could be purchased in a reasonable price without the need of their preparation. The factors manipulated in this protocol were chosen to enhance the efficiency of the banding patterns obtained and acquire superior degree of accuracy of bacterial typing. A comparison of both methods to facilitate the changes is seen in Table 23.

Step	Miranda *et al* method	Enhanced rapid method
Inoculation	Take a single colony from 16-18 hrs incubation. Inoculate in 5 mls BHI[1] broth without shaking at 37^{o}C	Take 5-10 colonies from 16-18 hrs incubation at 37^{o}C
Cell suspension preparation	Centrifuge at 4000 × *g* for 20 minutes & resuspend pellets in 2 mls PIV buffer (1 M tris, 1 M NaCl, pH 7.6). Allow to equilibrate at 50^{o}C	Emulsify colonies in 2 mls 1X PBS.
Plug preparation	1.6% LMP 2 mls	1.6% LMP with 1% SDS
Lysis steps	Lyse plugs in lysis solution (1 M Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij[58], 0.2% deoxycholate, 0.5% Na lauroylsarcosine, 50 µg ribonuclease A + 1 mg/ml lysozyme, pH 7.6). Incubate at 37^{o}C overnight. Incubate with ESP[2] buffer (0.4 M EDTA, 1% SDS, 0.5 mg/ml PK) for 24 hrs at 50^{o} C	Lyse plugs in 2 mls CLS (50 mM Tris, 50 mM EDTA, 2.5 mg/ml lysozyme, 1.5 mg/ml PK, pH8). Incubate for one hour at 55^{o}C
Washing Steps	Wash once with 1X TE buffer with 1 mM PMSF at 37^{o}C Wash 3X with with 0.1X TE buffer for half an hour at 37^{o}C	Wash once with pre-heated 1X TE buffer with 1 mM PMSF, then twice with pre-heated 0.1X TE buffer all for 15 minutes in a shaking water bath at 55^{o}C

Table 23: Comparison between Miranda *et al* and the enhanced PFGE protocol.

4.4 Sequencing with plasmid and genetic environment studies

4.4.1 Selection of strains for the detection of the most prevalent CTX-M family member

Using primers described by Dutour (2002), all of the isolates listed in Table 24 yielded a product size of 903 bp (Figure 18). Sequences obtained from sequencing facilities were identified by sequence alignment BLAST. A summary of the sequencing results are provided in Table 22. It was found that all of the strains selected, except for 86, 89, 97, and 102 shared ≥ 98% identity with *bla*$_{\text{CTX-M-15}}$ (GenBank accession № HQ157357.1). Other CTX-Ms found upon sequencing were *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{CTX-M-28}}$, *bla*$_{\text{CTX-M-55}}$, and *bla*$_{\text{CTX-M-117}}$ corresponding to isolate number 102, 86, 89, and 97 respectively. The nucleotide sequences of all of the reported *bla*$_{\text{CTX-M}}$ genes are listed in Appendix (A). The amino acid sequences of all of the reported *bla*$_{\text{CTX-M}}$ genes are listed in Appendix (B).

The strains that shared 100% similarity with *bla*$_{\text{CTX-M-15}}$ (GenBank accession number HQ157357.1), were considered to share the same progenitor and acquire *bla*$_{\text{CTX-M-15}}$ in the same phase (strain 4, 12, 17, 20, 37, 47, 55, 57, 60, 61, 74, 88, 100, 101, and 105) as seen in (Table 24). In contrast, the rest of the strains that share 98-99% with *bla*$_{\text{CTX-M-15}}$ (GenBank accession number HQ157357.1), are thought to be from different origins. The amino acid sequences of the strains sharing 98-99% similarity with *bla*$_{\text{CTX-M-15}}$, were compared together (Figure 19). From the sequencing and the alignment results of *bla*$_{\text{CTX-M-15}}$, it was found out that there are three clones present

all from different origins. Strains sharing 100% similarity with the nucleotide and amino acid sequence of *bla*$_{CTX\text{-}M\text{-}15}$ (strain 4, 12, 17, 20, 37, 47, 55, 57, 60, 61, 74, 88, 100, 101, and 105) suggest that they originate from the same source (15 strains). While strains sharing 96 and 99% protein sequence similarity with *bla*$_{CTX\text{-}M\text{-}15}$ (Strain 75, 80, 82, 87, 90, 91, 92, 93, 94, 95, 96, 98, and 99) were analysed based on the nucleotide base. From the multi-alignment results (Figure 14), it was found that these strains probably derived from two origins; just one strain from a single progenitor (strain 99) and 12 strains from a different origin (strains 5, 80, 82, 87, 90, 91, 92, 93, 94, 95, 96, and 98) (Figure 19). The results point out to the presence of silent, synonomous mutations, where mutations only occur on the nucleotide level and are not reflected by changes in the amino acid and therefore the protein structure or function. In other words, the findings suggest the existence of three different progenitors and the acquisition of *bla*$_{CTX\text{-}M\text{-}15}$ genes in three different phases. Also, we can conclude that *bla*$_{CTX\text{-}M\text{-}15}$ is the most prevalent CTX-M member in all of the five hospitals in Kuwait, specifically in strains with MDR pattern.

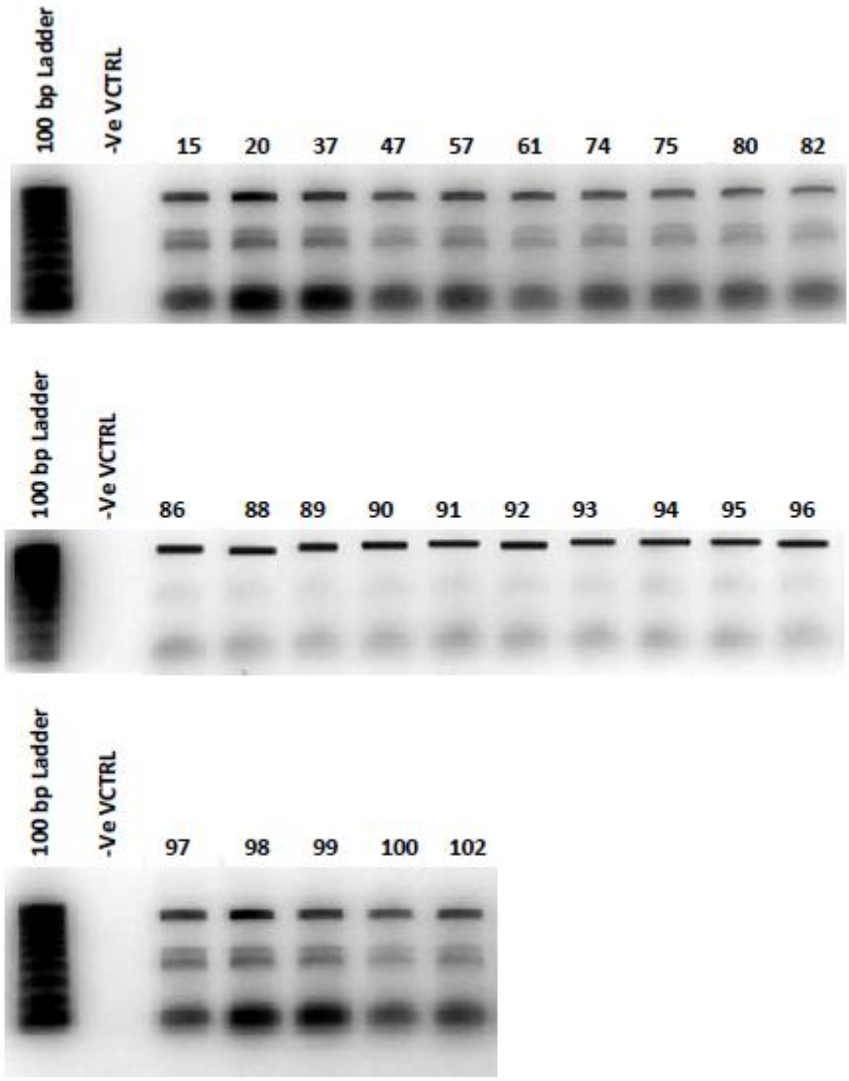


Figure 18: PCR products with primers described by (Dutour *et al.*, 2002). The expected product size is 903 bp. -ve CTRL indicates negative control, 15, 20, 37, 47, 57, 61, 74, 75, 80, and 82, 86, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, and 102 represent isolate numbers.

Isolate №	Hospital	Strain	Gene detected	Gene accession №	%similarity
4	M	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
15	M	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
17	M	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
20	M	*K. pneumoniae*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
37	M	*K. pneumoniae*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
47	M	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
55	K	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
57	K	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
60	K	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
61	K	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
74	ID	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
75	ID	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
80	ID	*K. pneumoniae*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
82	ID	*K. pneumoniae*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
86	ID	*E. coli*	$bla_{\text{CTX-M-28}}$	EU531510.1	98%
87	ID	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
88	A	*K. pneumoniae*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
89	A	*K. pneumoniae*	$bla_{\text{CTX-M-55}}$	HM748991.1	98%
90	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
91	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
92	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
93	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
94	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
95	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
96	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
97	A	*E. coli*	$bla_{\text{CTX-M-117}}$	JN227085.1	98%
98	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	99%
99	A	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	96%
100	IB	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
101	IB	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%
102	IB	*E. coli*	$bla_{\text{CTX-M-3}}$	GQ339102.2	95%
105	IB	*E. coli*	$bla_{\text{CTX-M-15}}$	HQ157357.1	100%

Table 24: PCR sequencing and amplification results.

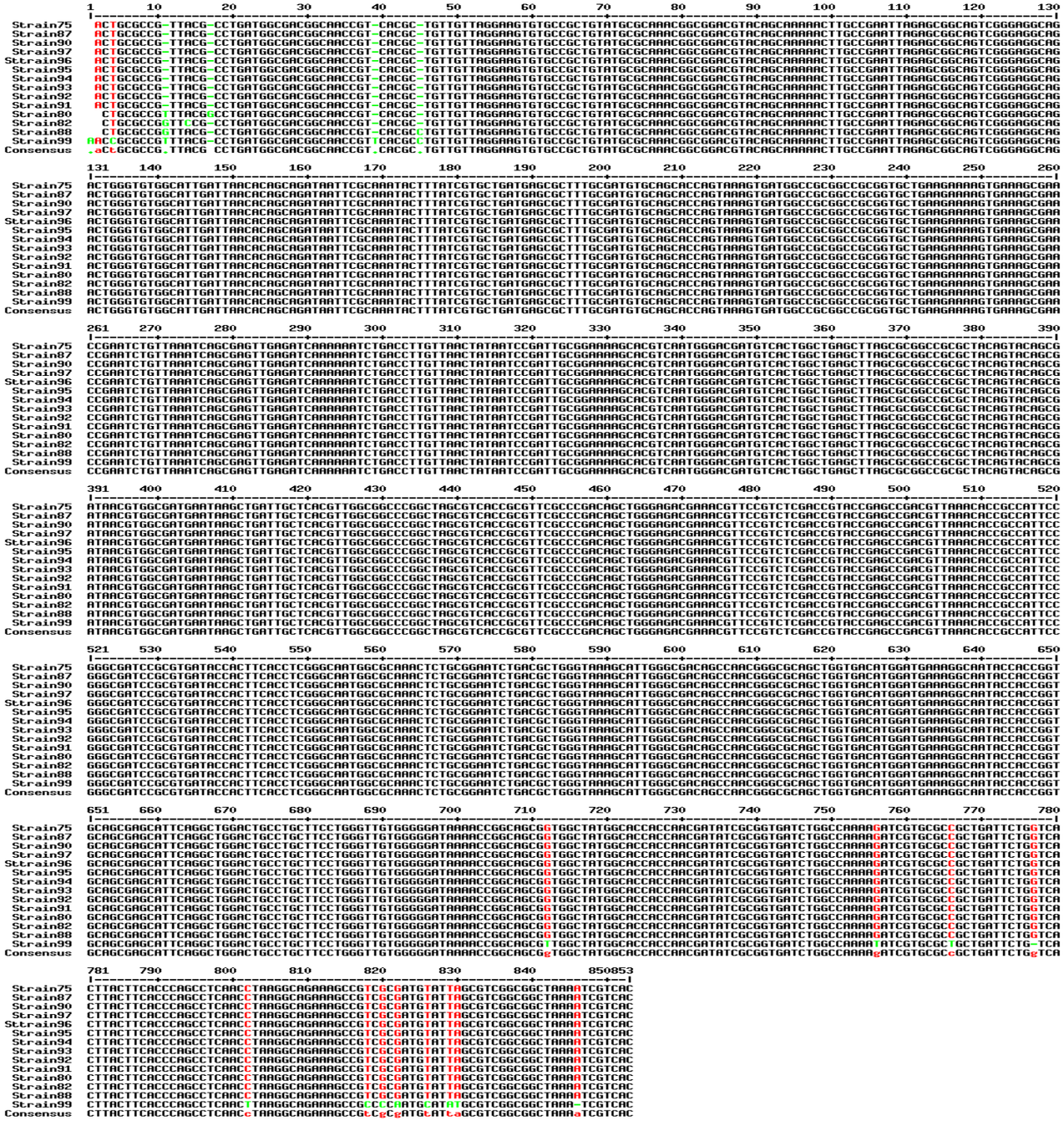


Figure 19: Nucleotide differences between isolates sharing 98-99% similarity with *bla*$_{\text{CTX-M-15}}$ sequences. Areas in black are identical, red indicates low consensus, and green indicates neutral consensus.

The alignment of the protein sequences of *bla*$_{CTX\text{-}M\text{-}15}$ can be seen in Figure 20. The alignment of the protein sequences of *bla*$_{CTX\text{-}M\text{-}28}$ (Figure 21). The alignment of the protein sequences of *bla*$_{CTX\text{-}M\text{-}55}$ can be seen in Figure 22. The alignment of the protein sequences of *bla*$_{CTX\text{-}M\text{-}117}$ (Figure 23). The alignment of the protein sequences of *bla*$_{CTX\text{-}M\text{-}3}$ can be seen in Figure 24.

Moreover, all of the derivatives of *bla*$_{CTX\text{-}M\text{-}15}$ gene were detected in the Al-Amiri hospital except for *bla*$_{CTX\text{-}M\text{-}28}$ which was found in Infectious Diseases hospital. The detection of *bla*$_{CTX\text{-}M\text{-}3}$ in Ibn-Sina hospital was from an isolate collected earlier in 2006.

When aligning the amino acid sequences of the reported *bla*$_{CTX\text{-}M}$ genes (*bla*$_{CTX\text{-}M\text{-}3}$, *bla*$_{CTX\text{-}M\text{-}15}$, *bla*$_{CTX\text{-}M\text{-}28}$, *bla*$_{CTX\text{-}M\text{-}55}$, and *bla*$_{CTX\text{-}M\text{-}117}$) (Figure 25), the differences laid at positions 80, 177, 242, and 288. It is thought that *bla*$_{CTX\text{-}M\text{-}15}$ is derived from *bla*$_{CTX\text{-}M\text{-}3}$, where there is only one amino acid change laying at position 242 (Asp-240→Gly) (Baraniak *et al.*, 2002, Cartelle *et al.*, 2004, Girlich *et al.*, 2009, Karim *et al.*, 2001, Novais *et al.*, 2007, Novais *et al.*, 2008, Poirel *et al.*, 2002a). CTX-M-28 is less common than CTX-M-15 but suggests in vivo mutation of CTX-M-15 (Dubois *et al.*, 2010, Menezes *et al.*, 2010). In *bla*$_{CTX\text{-}M\text{-}28}$, Aspartate at position 288 is substituted by Asparagine (Asp-288→Asn) (Dubois *et al.*, 2010) (Table 25). This difference has been ignored by many researchers and therefore *bla*$_{CTX\text{-}M\text{-}28}$ can be misinterpreted as *bla*$_{CTX\text{-}M\text{-}15}$ (Menezes *et al.*, 2010). On the other hand, *bla*$_{CTX\text{-}M\text{-}55}$ is

different from *bla*$_{CTX-M-15}$ by one amino acid (i.e. valine for alanine) at position 80 (Kiratisin *et al.*, 2007) (Table 25). This work describes the first report of *bla*$_{CTX-M-55}$ in the Middle East area. Also, this work reports the first description of *bla*$_{CTX-M-117}$ in Kuwait, which differs from *bla*$_{CTX-M-15}$ by one amino acid change at position 177 (i.e. glutamine for proline). All the differences of the reported CTX-Ms are noted in Table 25.

CTX-M-type	Variation at position 80	Variation at position 177	Variation at position 242	Variation at position 282
CTX-M-3	Alanine	Proline	**Aspartic acid**	**Aspartic acid**
CTX-M-15	Alanine	Proline	Glycine	**Aspartic acid**
CTX-M-28	Alanine	Proline	Glycine	**Asparagine**
CTX-M-55	**Valine**	Proline	Glycine	**Aspartic acid**
CTX-M-117	Alanine	**Glutamine**	Glycine	**Aspartic acid**

Table 25: Amino acid changes of the reported CTX-Ms genes in this study.

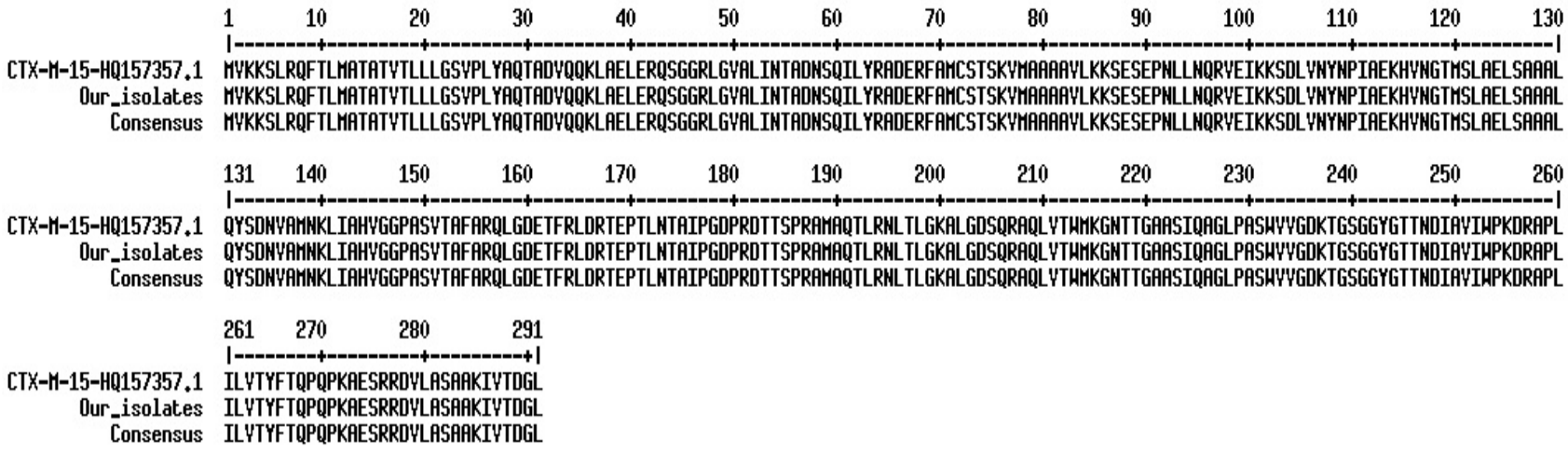


Figure 20: Alignment of CTX-M-15 protein with the translation of the amino acid sequence obtained from our strains. The black coloured letters indicate that both sequences share high consensus. The proteins sequences were aligned using multi-align website http://multalin.toulouse.inra.fr/multalin/.

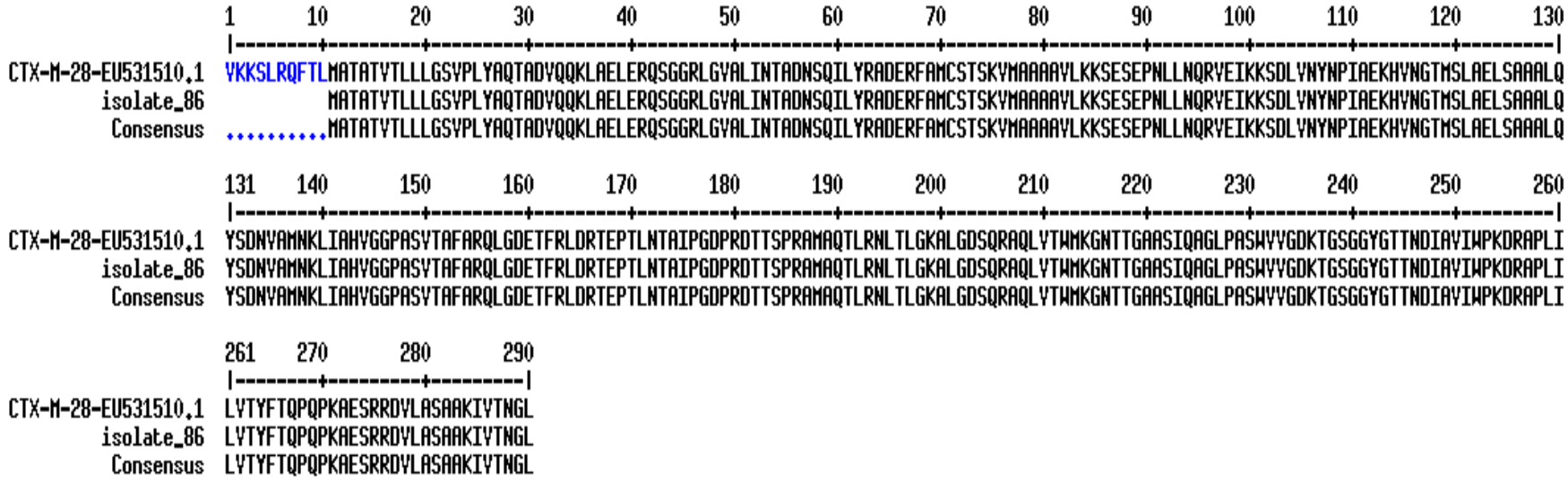


Figure 21: Alignment of CTX-M-28 protein with the translation of the amino acid sequence obtained from isolate 86. The black coloured letters indicate that both sequences share high consensus, the blue coloured letters indicate neutral consensus. The proteins sequences were aligned using multi-align website http://multalin.toulouse.inra.fr/multalin/.

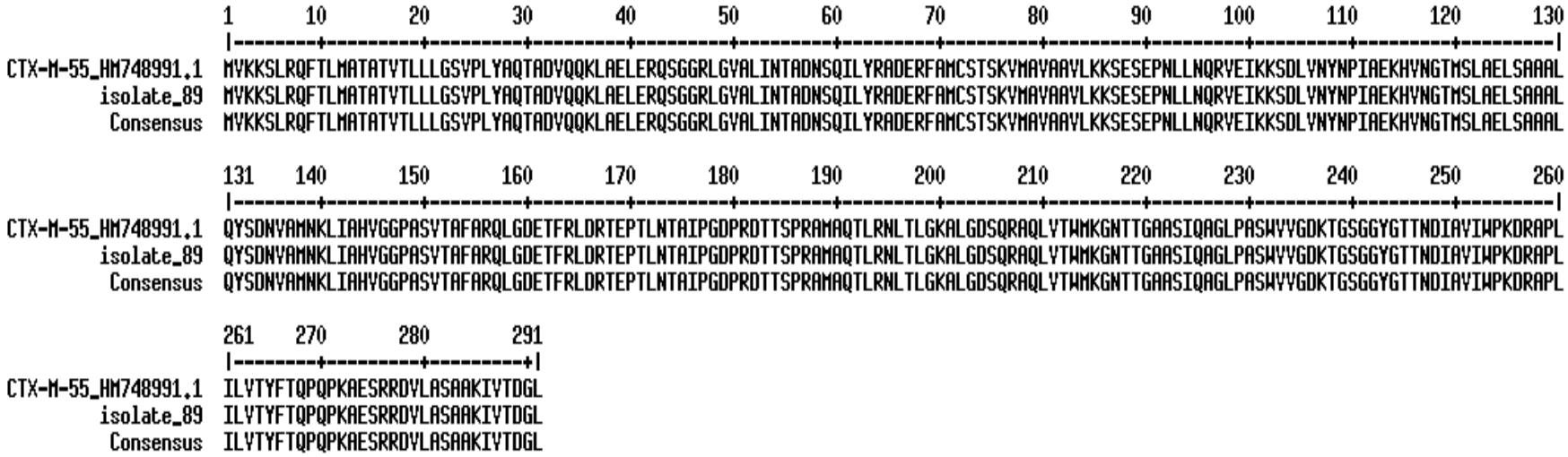


Figure 22: **Alignment of CTX-M-55 protein with the translation of the amino acid sequence obtained from isolate 89.** The black coloured letters indicate that both sequences share high consensus. The proteins sequences were aligned using multi-align website http://multalin.toulouse.inra.fr/multalin/.

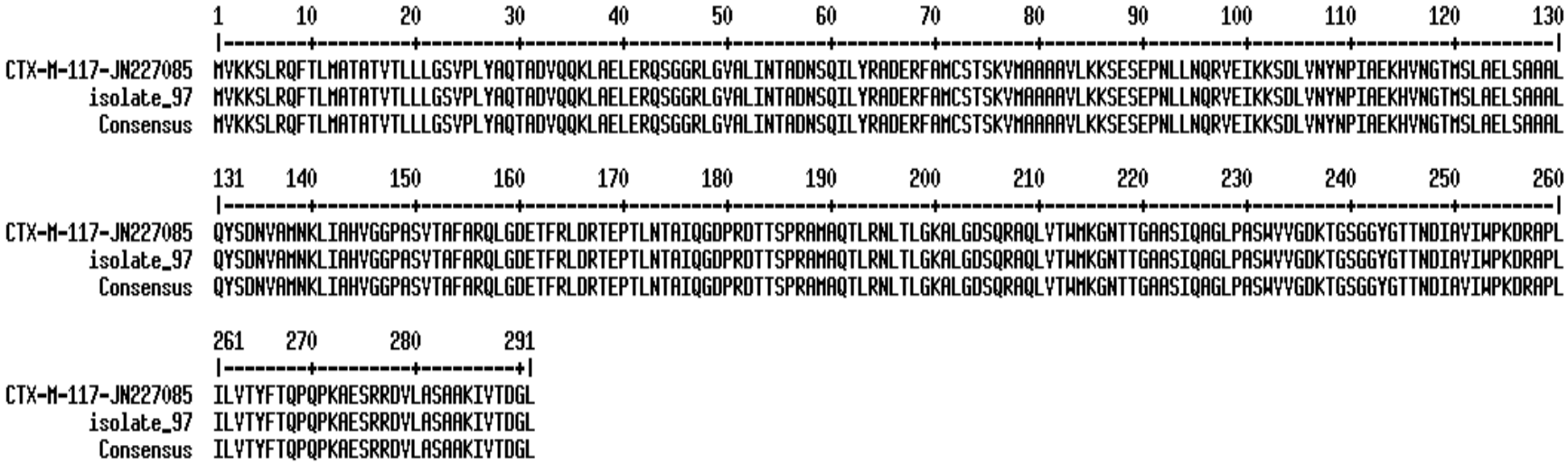


Figure 23: Alignment of CTX-M-117 protein with the translation of the amino acid sequence obtained from isolate 97. The black coloured letters indicate that both sequences share high consensus. The proteins sequences were aligned using multi-align website http://multalin.toulouse.inra.fr/multalin/.

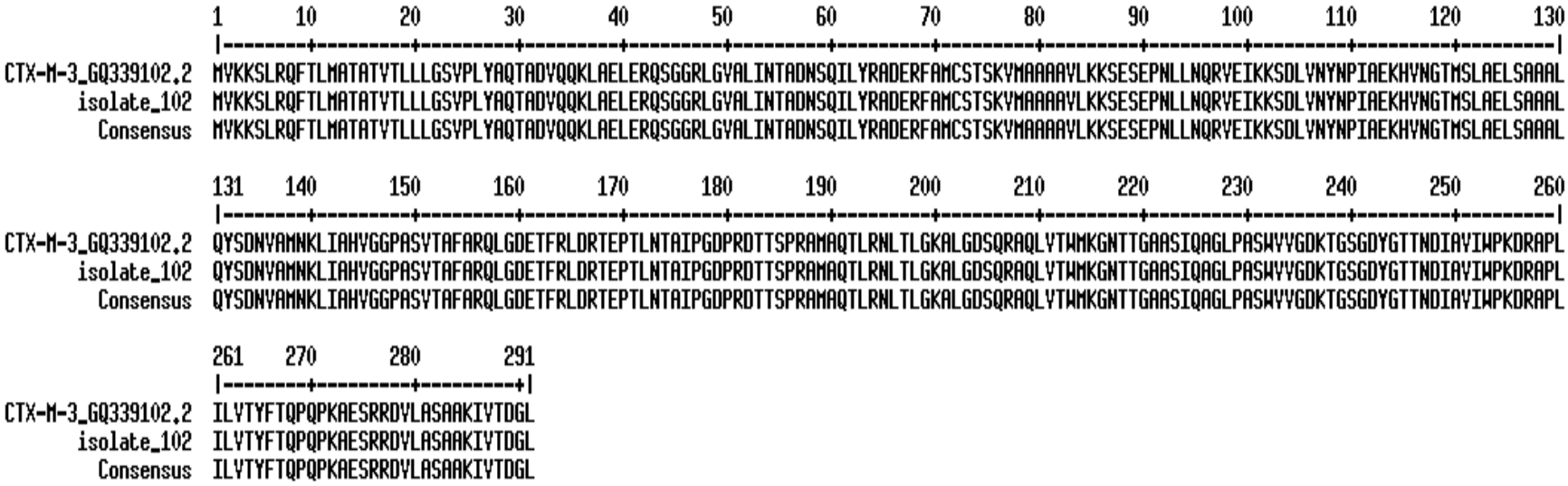


Figure 24: Alignment of CTX-M-3 protein with the translation of the amino acid sequence obtained from isolate 102. The black coloured letters indicate that both sequences share high consensus. The proteins sequences were aligned using multi-align website http://multalin.toulouse.inra.fr/multalin/.

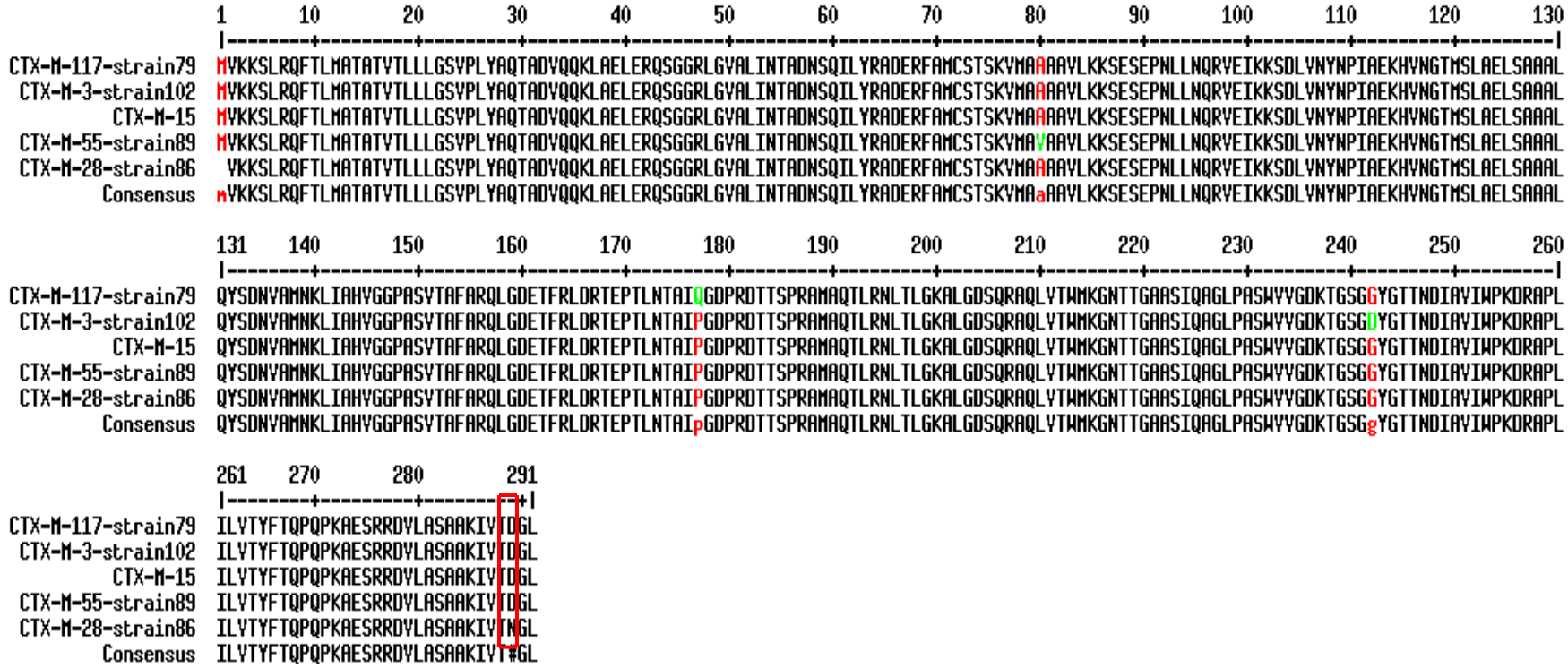


Figure 25: Alignment of the amino acid sequence of all of the CTX-Ms found in this study. The letters represented in black colour are the same amino acids found in all of the CTX-Ms reported. The letters in red and green colour are the amino acids that are different in the reported CTX-Ms. The changes between CTX-M-28 and the rest are outlined in the red box.

4.4.2 Selection of strains for plasmid and genetic environment studies

Twenty-five strains were chosen for the comparison of the genetic environment and the detection of possible common insertion sequences or other common transpositional elements. Also, the 25 strains were used for further plasmid studies. Plasmid studies included the detection of the plasmid sizes carrying the $bla_{CTX\text{-}M}$ genes detected and the Incompatibility grouping of the very same plasmids. A list of the twenty-five strains used for this part of the study is summarized in Table 11.

In terms of PFGE, The selected *E. coli* and *K. pneumoniae* strains for genetic environment studies were unrelated except for six strains (91 and 92, 95 and 96, and 97 and 98) as seen in Figure 26. Thirteen of the selected *E. coli* strains were unrelated and their similarity values ranged from 21.5% to 72.7%. Two of the remaining six *E. coli* strains proved to be similar (91 and 92) and four were identical (95 with 96 and 97 with 98) (Figure 26). On the other hand, no epidemiological relationship was seen in *K. pneumoniae* strains chosen for the genetic environment studies; their similarity values ranged from 29 to 67% (Figure 27). Importantly, all of the similar and identical strains were from the same hospital (i.e. Al-Amiri hospital) (Figure 26).

In other words, the absence of clonal relationship among most of the collected strain led to selection on the basis of MDR patter, with the finding of new $bla_{CTX\text{-}M}$

genes reported as in strain 86, 89, 97, and 102. These strains encode *bla*$_{\text{CTX-M-28}}$, *bla*$_{\text{CTX-M-55}}$, *bla*$_{\text{CTX-M-117}}$, and *bla*$_{\text{CTX-M-3}}$ respectively.

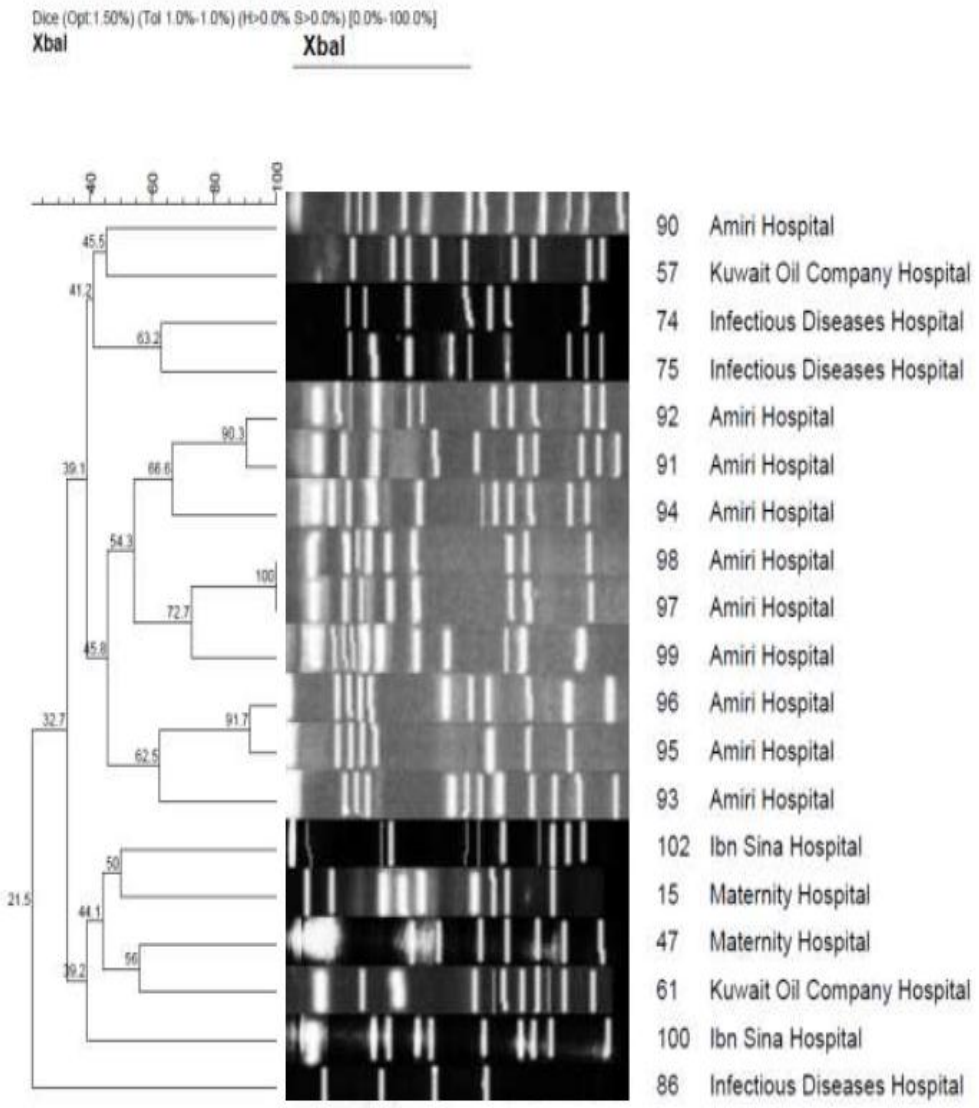


Figure 26: PFGE banding patterns obtained for strains selected for genetic environment studies. *E. coli* strains.

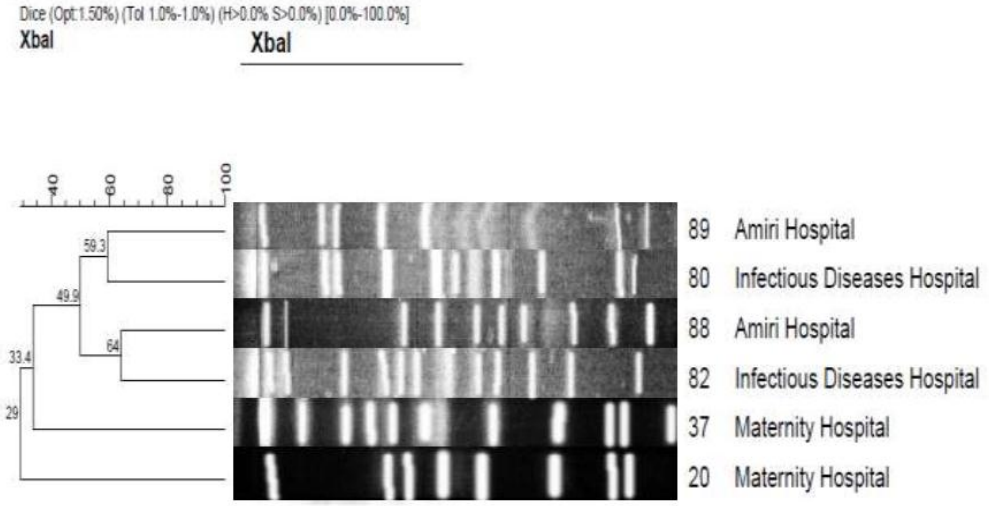
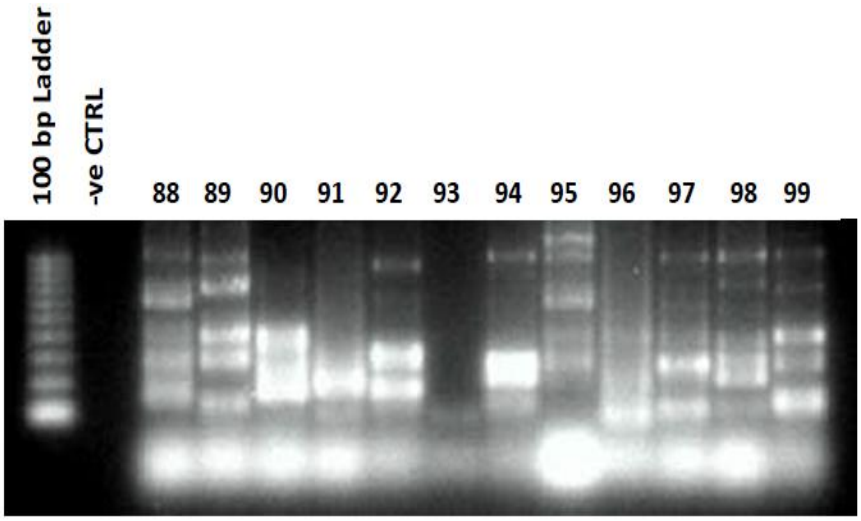


Figure 27: PFGE banding patterns obtained for strains selected for genetic environment studies. *K. pneumoniae* strains.

The results of the PCRs done for the upstream genetic environment detection are seen in Figure 28. Genome-walking PCR succeeded in obtaining the upper flanking regions of all of the strains. Regardless, GW-PCR failed to detect any regions for the downstream genetic context of the selected strains. Rather, simplex PCR was employed to detect the downstream area of the described $bla_{CTX\text{-}M}$ genes as seen in Figure 29.

From GW-PCR and simplex PCR, seven different genetic contexts were sought from the 25 strains selected for plasmid studies seen in Table 11. Both upstream and downstream arrangements of the reported CTX-M members are described in this work.

(a)



(b)

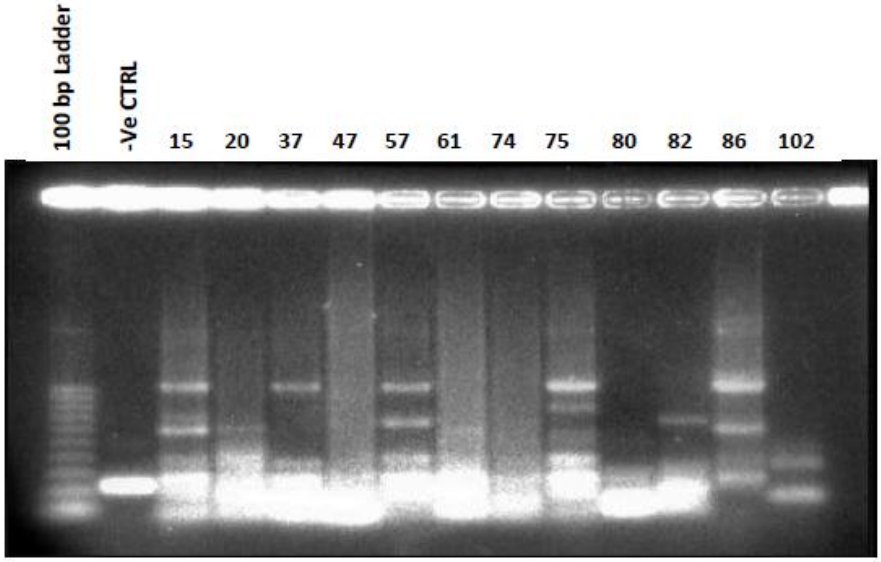
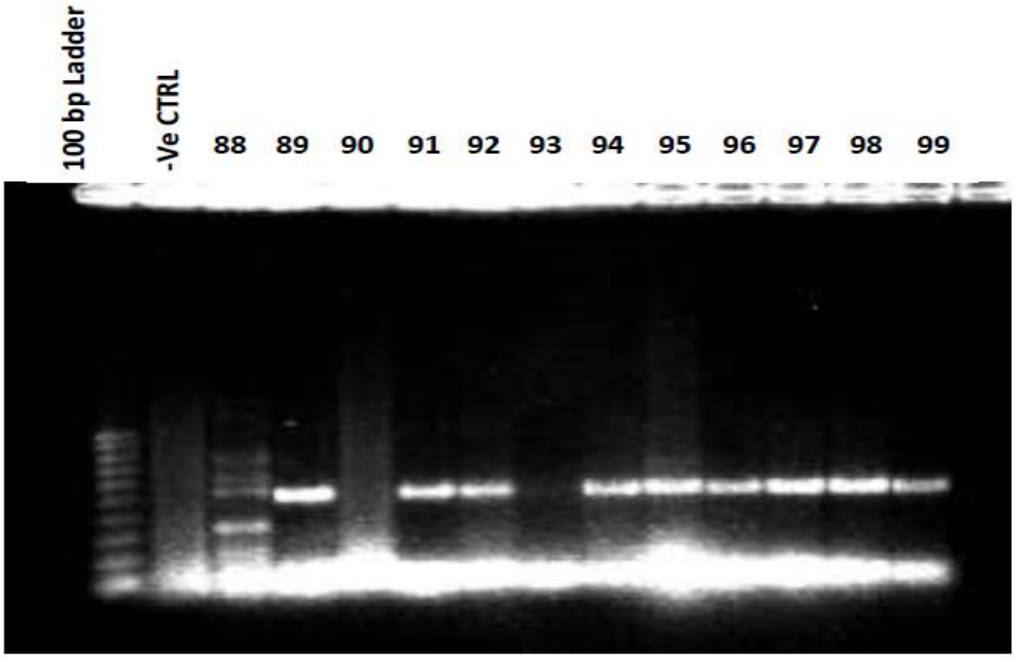


Figure 28: GW PCR for the upstream area. (a)Shows PCR products for strains collected from Al-Amiri hospital. (b) Shows PCR products for the rest of the strains selected for further detection of genetic context and plasmid studies.

(a)



(b)

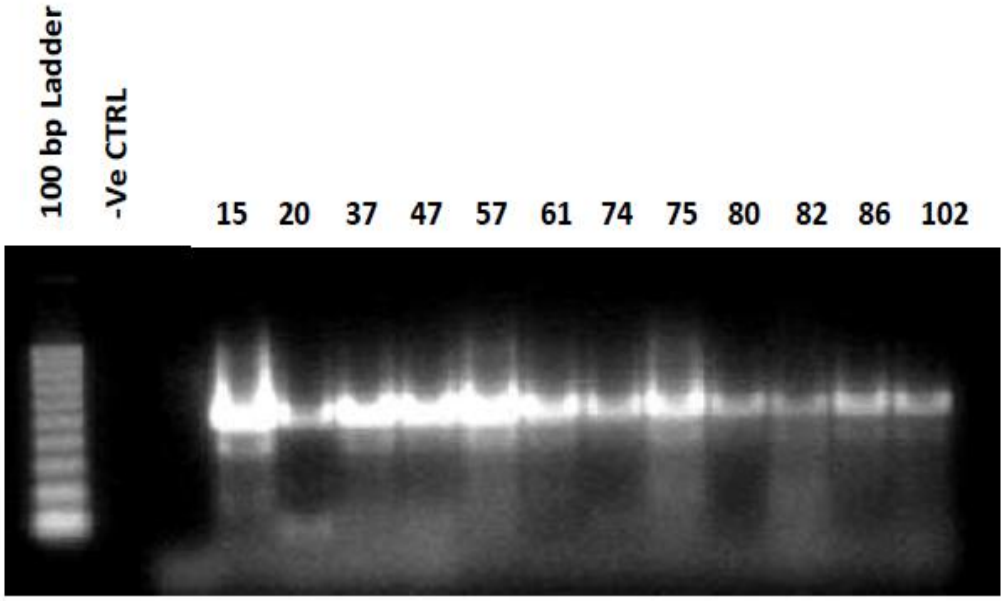
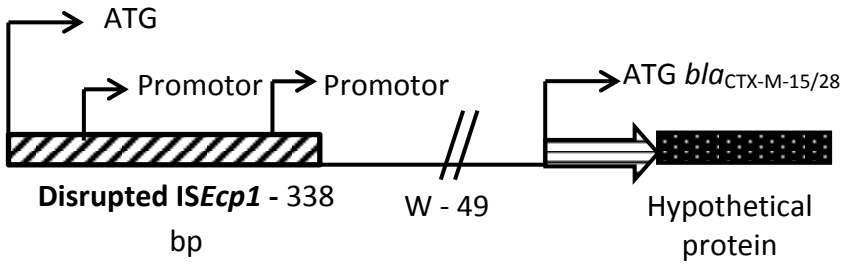
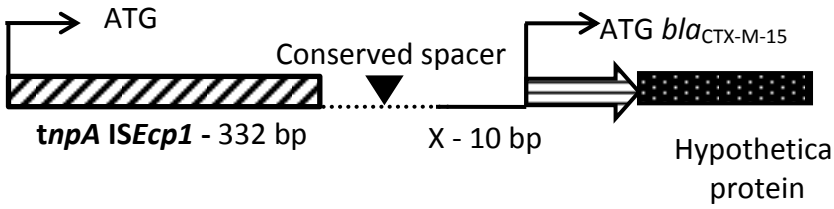


Figure 29: PCR Results for the downstream area. (a) Downstream PCR products for strains collected from Al-Amiri hospital. (b) Downstream PCR products for the remainder of the strains selected for plasmid and genetic environment studies.

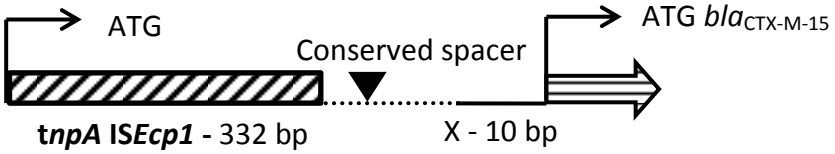
The downstream genetic environment of strain numbers 20, 90, and 93 could not be obtained. The majority of the strains selected for plasmid studies (numbers 37, 47, 91, 92, 95, 96, 99, and 100) shared the genetic context identified in D (Figure 30). This genetic arrangement was previously reported in *Acinetobacter baumannii* (*A. baumannii*) strain carrying $bla_{\text{CTX-M-15}}$ gene reported in Haiti (GenBank accession number JN788267.1) (Potron *et al.*, 2011). Nevertheless, out of the 25 strains selected for plasmid studies, six strains (strain number 15, 74, 75, 80, 82, and 86) shared the same genetic environment but with variable sizes of the hypothetical protein (463-471 bp) described downstream the $bla_{\text{CTX-M-15}}$ gene (A) (Figure 30). A similar genetic orientation was previously reported in an *E. coli* strain reported from the United Kingdom (GenBank accession number AY536259.1) (Woodford *et al.*, 2004). Different genetic arrangements were also found in the rest of the selected strains in One strains showed structure (B), one strains showed structure (C), eight strains had the genetic context of structure (D), two strains showed to have structure (E), also two strains were found to share structure (F), only one strains was found to have (G), and one strain as well had structure (H), one isolate showed to have the genetic context described in structure (I), and one isolate was found to have structure (J) (Figure 30).


ATG
Promotor
Promotor
ATG $bla_{CTX-M-15/28}$
Disrupted IS*Ecp1* - 338 bp
W - 49
Hypothetical protein

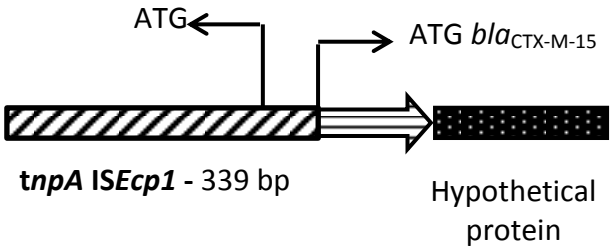
(A)


ATG
Conserved spacer
ATG $bla_{CTX-M-15}$
t*npA* IS*Ecp1* - 332 bp
X - 10 bp
Hypothetical protein

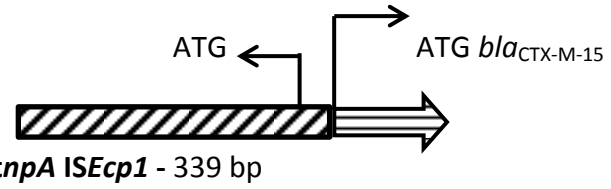
(B)


ATG
Conserved spacer
ATG $bla_{CTX-M-15}$
t*npA* IS*Ecp1* - 332 bp
X - 10 bp

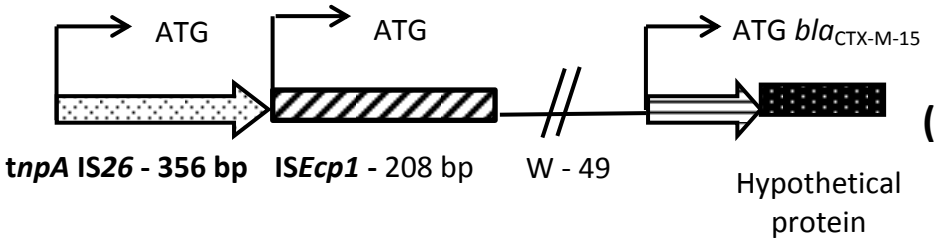
(C)


ATG
ATG $bla_{CTX-M-15}$
t*npA* IS*Ecp1* - 339 bp
Hypothetical protein

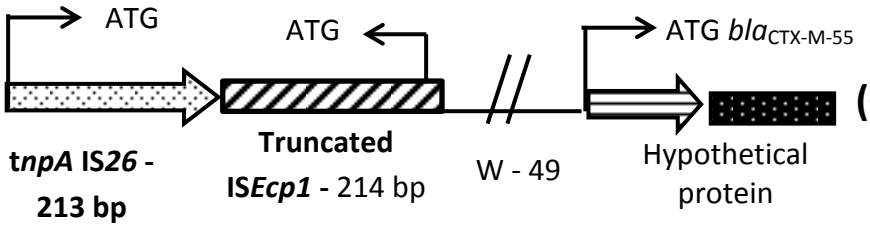
(D)


ATG
ATG $bla_{CTX-M-15}$
t*npA* IS*Ecp1* - 339 bp

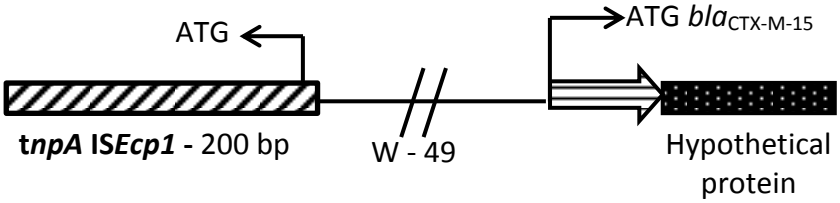
(E)


ATG
ATG
ATG $bla_{CTX-M-15}$
t*npA* IS*26* - 356 bp
IS*Ecp1* - 208 bp
W - 49
Hypothetical protein

(F)


ATG
ATG
ATG $bla_{CTX-M-55}$
t*npA* IS*26* - 213 bp
Truncated IS*Ecp1* - 214 bp
W - 49
Hypothetical protein

(G)


ATG
ATG $bla_{CTX-M-15}$
t*npA* IS*Ecp1* - 200 bp
W - 49
Hypothetical protein

(H)

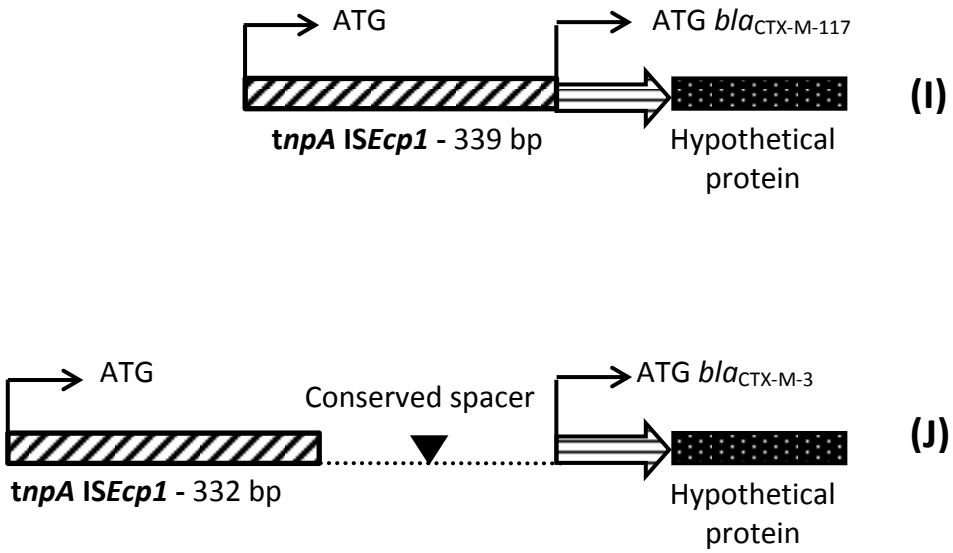


Figure 30: Genetic environments of all of the strains. The letters indicated refer to the genetic environment of strains ;(A) 15, 74, 75, 80, 82 and 86, (B) 20, (C) 88, (D) 37, 47, 91, 92, 95, 96, 99 and 100, (E) 90 and 93, (F) 57 and 61, (G) 89, (H) 94, (I) 97 and (J) 102.

4.4.3 Genome Walking and Simplex PCR for genetic environment studies

Upon sequencing the products of walking PCR experiment seen in Figure 28, IS*Ecp1* was noted to be present at the upper flanking regions of all of the described CTX-M genes (Figure 30). The existence of IS*Ecp1* in the selected strains was not conditional for the presence of the ''W'' region. Rather, IS*Ecp1* could exist on its own at the 5' terminus of the $bla_{CTX\text{-}M}$ genes. This can be seen in structure (B), (C), (D), (E), (I), and (J) in Figure 30, where IS*Ecp1* is either preceded by a 10 bp and a conserved spacer, $bla_{CTX\text{-}M}$ gene, or just by a conserved spacer. Notably, the distances of IS*Ecp1* from the start codons of the reported $bla_{CTX\text{-}M}$ genes are variable. Similarly, the sizes of IS*Ecp1* sequences are variable as well (Figure 30). The integrity of IS*Ecp1*, in this study, was often compromised. In some strains, a noticeable disruption of IS*Ecp1* is described as seen in structure A (Figure 30). A similar report was documented from a strain collected from the UK carrying $bla_{CTX\text{-}M}$ gene (Woodford *et al.*, 2004). However, in other strains the IS*Ecp1* is found to be truncated as seen in structure G (Figure 30). The direction of the orientation of IS*Ecp1* can be the same as that of the $bla_{CTX\text{-}M}$ gene described downstream the insertion sequence as seen in structures A, B, C, D, E, F, I, and J (Figure 30). In contrast, the IS*Ecp1* can be oriented in the opposite direction to that $bla_{CTX\text{-}M}$ gene as seen in structure G in Figure 30. A previous description of an inverse insertion of IS*Ecp1* was reported in an *E. coli* strain carrying $bla_{CTX\text{-}M\text{-}3}$ gene collected from Spain (GenBank accession number FJ235691.1) (Diestra *et al.*, 2009). The presence of

IS*Ecp1* can be accompanied by the existence of another insertion sequence. Most commonly, IS*26* is reported to co-exist with IS*Ecp1* at the upper flanking region of bla_{CTX-M} genes (Diestra *et al.*, 2009, Kiratisin *et al.*, 2007, Novais *et al.*, 2007). Likewise, IS*26* was detected upstream IS*Ecp1* as seen in structure F and G (Figure 30).

Interestingly, the upstream context of most of the strains collected from Al-Amiri hospital, carrying the $bla_{CTX-M-15}$ gene, share high similarity (95-98%) with the upper flanking region of an *A. baumannii* strain collected from Haiti (GenBank accession number JN788267.1) (Potron *et al.*, 2011). Moreover, two strains from the Maternity hospital (strain 37 and 47) and one strain from Ibn sina hospital (strain 100) show the same upstream genetic environment as seen in Table 26. This indicates the circulation of a certain plasmid carrying $bla_{CTX-M-15}$ gene around both hospitals. Another common upstream orientation is found among five strains from the Infectious Diseases Hospital (strain 74, 75, 80, 82, and 86) and one strain from the Maternity hospital (strain 15), sharing 99% similarity with the upstream genetic environment of an *E. coli* strain collected from the UK (Table 26) (Woodford *et al.*, 2004). A less common upstream orientation found among my strains (strain 20 and 88) was the same as that reported in an *Enterobacter aerogenes* (*E. aerogenes*) strain collected from Russia (GenBank accession number HQ214045.1) (Table 26).

Another less common organization of the upstream environment of the described *bla*$_{CTX-M}$ gene is seen in strain 57 and 61, collected from KOC hospital sharing 96% similarity with a *K. pneumoniae* strain collected from Hungary (GenBank accession number EU556755.1) (Table 26).

The upper flanking region of *bla*$_{CTX-M-55}$ was found to be unique to strain 89, sharing 93% similarity with the upper flanking region of an *E. coli* strain carrying *bla*$_{CTX-M-3}$ (GenBank accession number FJ235691.1) (Diestra *et al.*, 2009) as seen in Table 26. Another unique upstream structure was obtained from strain 102, carrying *bla*$_{CTX-M-3}$ gene, sharing 96% similarity with the upstream context of an *E. aerogenes* strain collected from Russia (GenBank accession number HQ214049.1) seen in (Table 26)

The genome walking PCR failed to detect the downstream genetic environment of the selected strains. Therefore, simplex PCR was used to provide an insight of the flanking downstream genetic orientation of the described *bla*$_{CTX-M}$ genes in this study. Using simplex PCR with primers described by (Sonnevend *et al.*, 2006), all of the selected strains listed in Table 11 provided a product size around 600 bp. The downstream area of the selected strains showed 99% similarity with a *K. pneumoniae* strain reported in Korea encoding *bla*$_{CTX-M-14}$ gene (GenBank accession number JQ343851.1) (Table 26). Interestingly, the hypothetical protein observed downstream the described *bla*$_{CTX-M}$ genes was with variable sizes (420-471 bp). Nevertheless, failure to maintain the genetic environment was faced in strain 88

and 90. A summary of the similarities of *bla*$_{CTX-M}$ genetic environment of Kuwaiti isolates with non-Kuwaiti isolates can be seen in (Table 26). A schematic drawing representing the seven different genetic environments obtained from this work is simplified in Figure 30.

Kuwait Strains		Similarity of the genetic environment							
		Upstream genetic environment similarity				Downstream genetic environment similarity			
Isolate №	bla_{CTX-M} gene and strains	GenBank accession №	% Identity	bla_{CTX-M} gene and strains	Country	GenBank accession №	% Identity	bla_{CTX-M} gene and strains	Country
15	CTX-M-15 *E. coli*	AY536259.1	99%	CTX-M *E. coli*	UK	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
20	CTX-M-15 *K. pneumoniae*	HQ214045.1	100%	CTX-M-15 *E. aerogenes*	Russia	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
37	CTX-M-15 *K. pneumoniae*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
47	CTX-M-15 *E. coli*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
57	CTX-M-15 *E. coli*	EU556755.1	96%	CTX-M-15 *K. pneumoniae*	Hungary	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
61	CTX-M-15 *E. coli*	EU556755.1	96%	CTX-M-15 *K. pneumoniae*	Hungary	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
74	CTX-M-15 *E. coli*	AY536259.1	99%	CTX-M *E. coli*	UK	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
75	CTX-M-15 *E. coli*	AY536259.1	99%	CTX-M *E. coli*	UK	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
80	CTX-M-15 *K. pneumoniae*	AY536259.1	99%	CTX-M *E. coli*	UK	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
82	CTX-M-15 *K. pneumoniae*	AY536259.1	99%	CTX-M *E. coli*	UK	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
86	CTX-M-28 *E. coli*	AY536259.1	99%	CTX-M *E. coli*	UK	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
88	CTX-M-15 *K. pneumoniae*	HQ214045.1	100%	CTX-M-15 *E. aerogenes*	Russia	Could not be detected			
89	CTX-M-55 *K. pneumoniae*	FJ235691.1	93%	CTX-M-3 *E. coli*	Spain	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
90	CTX-M-15 *E. coli*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	Could not be detected			
91	CTX-M-15 *E. coli*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea

Kuwait Strains		Similarity of the genetic environment							
		Upstream genetic environment similarity				Downstream genetic environment similarity			
Isolate №	***bla*$_{CTX-M}$ gene and strains**	**GenBank accession №**	**% Identity**	***bla*$_{CTX-M}$ gene and strains**	**Country**	**GenBank accession №**	**% Identity**	***bla*$_{CTX-M}$ gene and strains**	**Country**
92	CTX-M-15 *E. coli*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
93	CTX-M-15 E. coli	JN788267.1	98%	CTX-M-15 A. baumannii	Haiti	Could not be detected			
94	CTX-M-15 *E. coli*	FR717894.1	95%	CTX-M-15 *E. coli*	Argentine	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
95	CTX-M-15 *E. coli*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
96	CTX-M-15 *E. coli*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
97	CTX-M-117 *E. coli*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
99	CTX-M-15 *E. coli*	JN788267.1	98%	CTX-M-15 *A. baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
100	CTX-M-15 *E. coli*	JN788267.1	96%	CTX-M-15 *Acinetobacter baumannii*	Haiti	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea
102	CTX-M-3 *E. coli*	HQ214049.1	96%	CTX-M-3 *Enterobacter aerogenes*	Russia	JQ343851.1	99%	CTX-M-14 *K. pneumoniae*	Korea

Table 26: Similarity of *bla*$_{CTX-M}$ genes genetic environments of Kuwaiti isolates with non-Kuwaiti isolates.

4.4.4 Plasmid sizing and grouping

Using S1 nuclease to restrict the PFGE plugs, the numbers and sizes of the plasmids in the selected strains were determined. After separating the plasmids, gel extraction kits (Qiagen) were used to slice out the plasmids from the gels and extract the DNA for further amplification and sequencing with primers described by (Dutour *et al.*, 2002). These primers confirmed the sizes of the plasmids carrying the described $bla_{CTX\text{-}M}$ genes. Further amplification with primers described by (Carattoli *et al.*, 2005) allowed the identification of the Incompatibility groups of the plasmids carrying the described $bla_{CTX\text{-}M}$ genes. The plasmids sizes encoding $bla_{CTX\text{-}M}$ genes ranged from 60 to 270 Kb as seen in Table 27. One of the selected strains (74) was found to have three plasmids, of different sizes (62, 154, and 270 Kb), each carrying the $bla_{CTX\text{-}M\text{-}15}$ gene. Some strains (61 and 94) had two plasmids both encoding $bla_{CTX\text{-}M\text{-}15}$ gene. Another strain (88) had two plasmids, but only one was shown to carry the $bla_{CTX\text{-}M\text{-}15}$ gene. The plasmid size carrying $bla_{CTX\text{-}M\text{-}15}$ gene was determined to be 133kb. Two strains from Al-Amiri hospital (strain 90 and 93) had plasmid DNA visible on gels, but when the DNA was extracted from the gel and amplified with primers, no products were obtained. This points out to possible existence of $bla_{CTX\text{-}M\text{-}15}$ gene on the chromosome. Most of the plasmids could not have their incompatibility group determined. Yet, three of which showed to belong to IncFII as seen in strain 91, 94, and 99. All of the results of plasmid sizes and incompatibility groups of the selected strains for plasmid studies can be seen in Table 27.

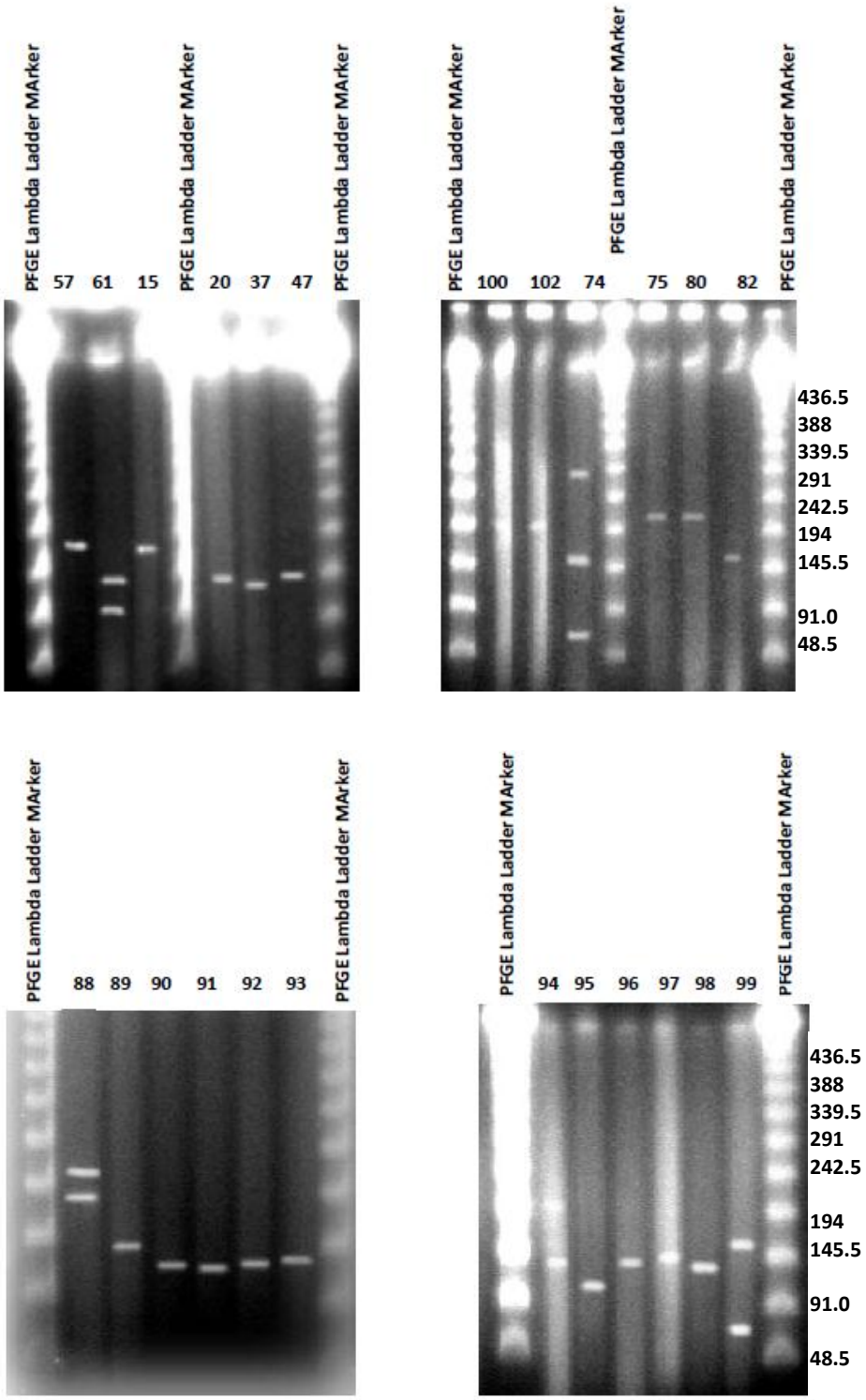


Figure 31: Sizing of plasmids obtained from strains selected for plasmid studies using s1 nuclease.

Isolate №	Hospital	*bla*$_{CTX-M}$ gene	Strains	Gene location	Plasmid size (Kb)	Inc grouping (Inc)
15	M	CTX-M-15	*E. coli*	Plasmid	166	-
20	M	CTX-M-15	*K. pneumoniae*	Plasmid	134	-
37	M	CTX-M-15	*K. pneumoniae*	Plasmid	127	-
47	M	CTX-M-15	*E. coli*	Plasmid	138	-
57	K	CTX-M-15	*E. coli*	Plasmid	166	-
61	K	CTX-M-15	*E. coli*	2 Plasmids	134 102	-
74	ID	CTX-M-15	*E. coli*	3 Plasmids	270 154 62	-
75	ID	CTX-M-15	*E. coli*	Plasmid	217	-
80	ID	CTX-M-15	*K. pneumoniae*	Plasmid	217	-
82	ID	CTX-M-15	*K. pneumoniae*	Plasmid	162	-
86	ID	CTX-M-28	*E. coli*	Plasmid		-
88	A	CTX-M-15	*K. pneumoniae*	plasmid	161	-
89	A	CTX-M-55	*K. pneumoniae*	Plasmid	133	-
90	A	CTX-M-15	*E. coli*	Chromosome	-	-
91	A	CTX-M-15	*E. coli*	Plasmid	60	IncFII
92	A	CTX-M-15	*E. coli*	Plasmid	63	-
93	A	CTX-M-15	*E. coli*	Chromosome	-	-
94	A	CTX-M-15	*E. coli*	2 Plasmids	194 130	IncFII
95	A	CTX-M-15	*E. coli*	Plasmid	109	-
96	A	CTX-M-15	*E. coli*	Plasmid	130	-
97	A	CTX-M-117	*E. coli*	plasmid	138	-
99	A	CTX-M-15	*E. coli*	Plasmid	125	- IncFII
100	IB	CTX-M-15	*E. coli*	Plasmid	194	-
102	IB	CTX-M-3	*E. coli*	Plasmid	194	-

Table 27: Plasmid sizes and incompatibility grouping of the selected strains for plasmids studies.

4.4.5 Conjugation studies

Transconjugants were obtained from isolates (15, 89, 97 and 102) indicating the transferability of *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-55}$, and *bla*$_{CTX-M-117}$. After amplification with primers described by Dutour (2002), transconjugants obtained the same genes that had been found in their parent strains (strain 15, 89, 97, and 102). In the transconjugants, plasmids carrying the same *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-55}$, and *bla*$_{CTX-M-117}$ had the same sizes and incompatibility groups as their parental enzymes. This was confirmed when using primers described by Carattoli (2005). In (Table 27), the plasmids of obtained from the transconjugants were sized. The sizes of the plasmids detected in the transconjugants were also the same as their parent strains; that is 166, 133, 138, and 194 Kb. The incompatibility groupings provided were also the same.

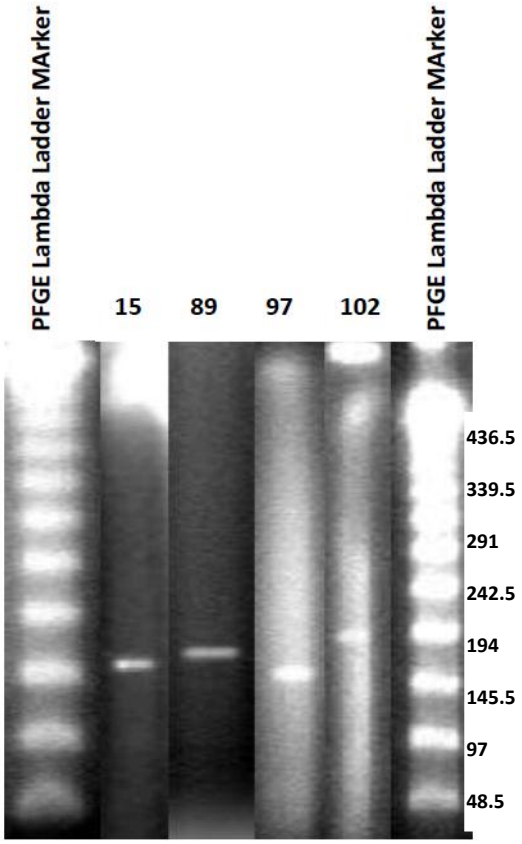


Figure 32: Sizing of plasmids obtained from transconjugants using S1 nuclease.

4.5 Examination of the structural models of CTX-Ms found

The examination of the structural models created showed that amino acids variations at positions 80, 177, 242, and 282 in *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-55}$, and *bla*$_{CTX-M-117}$ exerted effects on the structure of the enzyme and consequently its function. The variations among the reported CTX-Ms in this study are at the positions simplified in Table 25 were closer to the active site of the enzyme. The regions of the active site of all of the reported *bla*$_{CTX-M}$ enzymes and closer to the amino acid changes are Serine at position 70, lysine at position 73, serine at position 130, and glycine at position 158. The active site of the *bla*$_{CTX-Ms}$ is shown in Figure 33, Figure 34, Figure 35, Figure 36, and Figure 37. In *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-55}$, and *bla*$_{CTX-M-117}$, different amino-acids found at sites 80, 177, and 242 did confer different properties (Table 28).

Amino acid position	Amino acid	properties
78	Alanine	Hydrophobic
	Valine	Hydrophobic, aliphatic
177	Proline	Aliphatic, hydrophobic
	Glutamine	Negative, polar
242	Glycine	Hydrophobic
	Asprtate	Polar
288	Aspartate	Negative, polar
	Asparagine	Polar

Table 28: Properties of amino acids found at positions 78, 177, 242, and 288.

The molecular structure of CTX-M-15 is seen in Figure 33. Glycine at position 242 is the amino-acid variation of CTX-M-15 from CTX-M-3. In Figure 34, residues at

position 78 tend to be near to the bottom of the active site cleft. Not too far from the active lysine at position 73. In *bla*$_{CTX\text{-}M\text{-}55}$, valine sits at position 78, which resides on the base of the cleft, 8.48 Å distant from lysine of the active site. While in *bla*$_{CTX\text{-}M\text{-}15}$, valine is substituted for alanine residue. The change of valine residue for alanine acts to change the shape of the active site cleft by reducing the amount of space for substrate molecules to bind to the enzyme. This can be justified by the hydrophobicity of valine, making it exclusively situated on the interior of the protein. Pulling in of the cleft will consequently reduce the binding affinity of the enzyme to the substrate. This implies that the MICs of ceftazidime and cefoxitin in *bla*$_{CTX\text{-}M\text{-}55}$ are higher than those in *bla*$_{CTX\text{-}M\text{-}15}$ as shown in Table 29.

CTX-M	Strain	MICs of Ceftazidime (ml/L)	MICs of Cefoxitin (ml/L)
CTX-M-15	4, 15, 17, 20, 37, 47, 55, 57, 60, 61, 74, 75, 80, 82, 87, 88, 90, 91, 92, 93, 94, 95, 96, 98, 99, 100, 101, and 105	**32**	**2-128**
CTX-M-55	89	**64**	**64**

Table 29: A comparison of the MIC values of ceftazidime and cefoxitin in strains carrying *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{CTX\text{-}M\text{-}55}$.

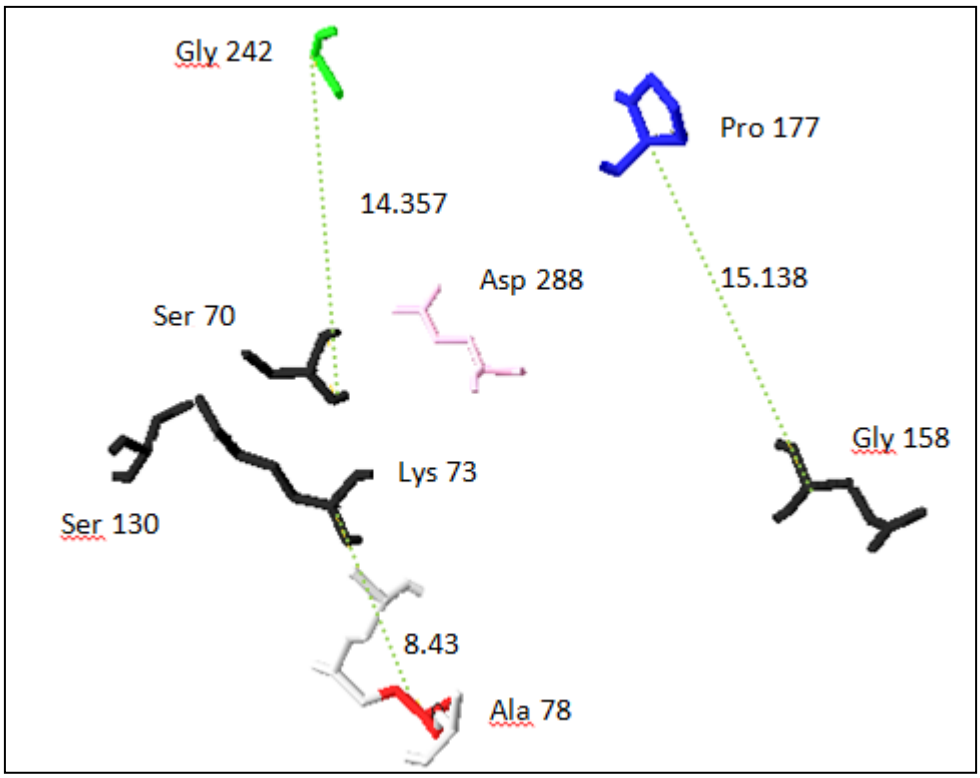


Figure 33: Glycine residue at position 242 in CTX-M-15. The active site of the enzyme is coloured in black. The structure was viewed in DeepView.

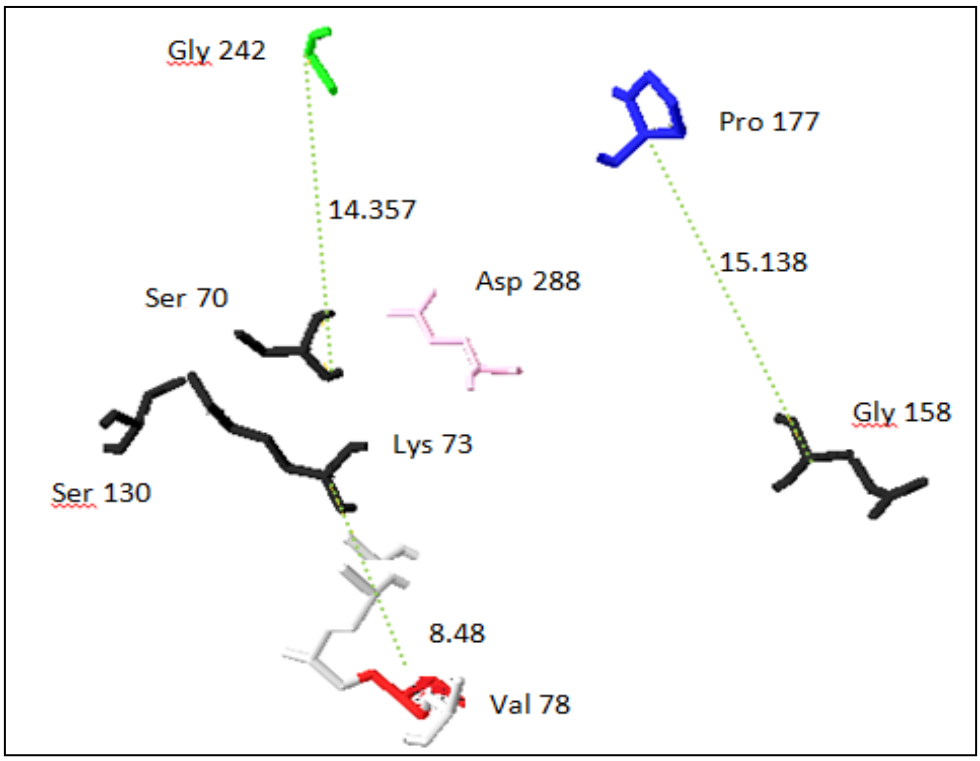


Figure 34: Valine residue at position 78 in CTX-M-55. The active site of the enzyme is coloured in black. The structure was viewed in DeepView.

In CTX-M-117 in Figure 35, glutamate at position 177 tends to form hydrogen bonds with serine present at the active site of the protein. This bonding is considered to be weak; still it would exert a considerable effect on the secondary structure of CTX-M-117 protein by enhancing the solubility of the protein in water. Accordingly, this enhances the ability of the molecule to bind to the drug. In CTX-M-15, proline at position 177, is more likely to be able to form stable tight direction changes in the enzymatic molecular structure. It is mainly accomplished by the propensity of proline to inhibit the formation of α-helices. This results in the narrowing of the enzyme's active site (i.e. glycine at position 158). Another property in which proline may impact on the active site is that it is largely aliphatic and hydrophobic. Most likely, proline is to be seen at regions of folding of the protein. Thus, it can be concluded that the presence of proline at this position of the enzyme decreases its affinity to the antimicrobial agent used (i.e. cephalosporins). As result, the values of the MICs to cephalosporins in strains carrying *bla*$_{\text{CTX-M-117}}$ should be lower than those in strains *bla*$_{\text{CTX-M-15}}$. This is indicated in Table 30

CTX-M	Strain	MICs of Ceftazidime (ml/L)	MICs of Cefoxitin (ml/L)
CTX-M-15	4, 15, 17, 20, 37, 47, 55, 57, 60, 61, 74, 75, 80, 82, 87, 88, 90, 91, 92, 93, 94, 95, 96, 98, 99, 100, 101, and 105	**32**	**2-128**
CTX-M-117	97	**8**	**8**

Table 30: A comparison of the MIC values of ceftazidime and cefoxitin in strains carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-117}}$.

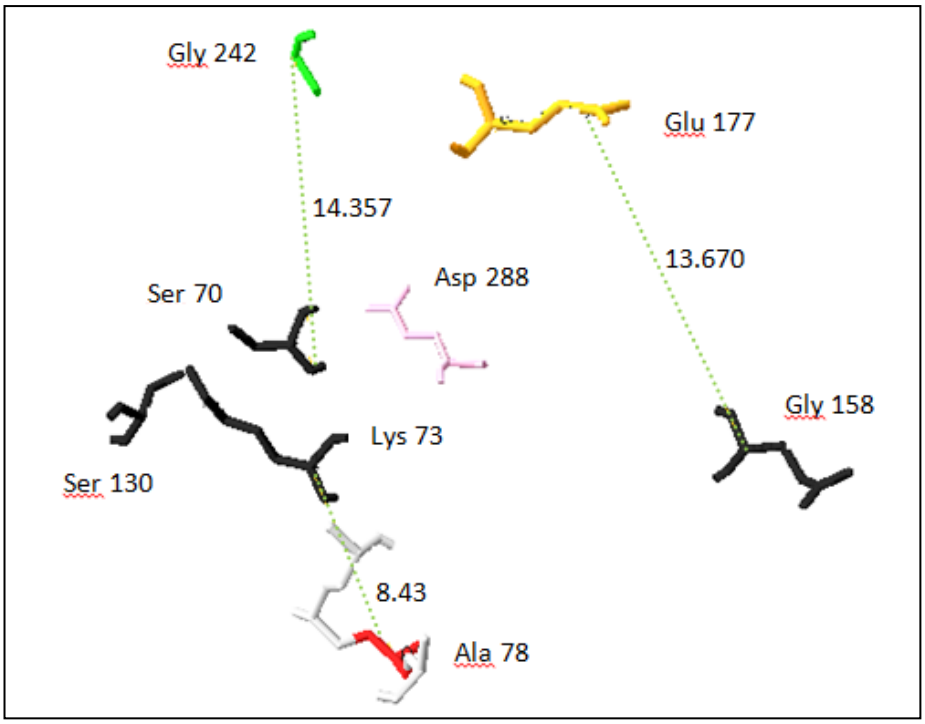


Figure 35: Glutamate residue at position 177 in CTX-M-117. The active site of the enzyme is coloured in black. The structure was viewed in DeepView.

In the structural model of CTX-M-3 seen in Figure 36, the variation of the amino acid at position 242 was found. CTX-M-3 has aspartate residue that is likely to decrease the substrate's affinity. Aspartate residue at position 242 is substituted for glycine in CTX-M-15. This is likely to result in decrease in the MIC values towards cephalosporins as seen in Table 31. Aspartate residue is closer to the active site serine at position 70; it is a very hydrophilic residue and contains a negative charge at neutral pH, making it predominantly found at the surface of the protein. Additionally, aspartate exhibits a great ability to form ionic bonds with other amino acids. Certainly, with serine present at position 70 at the active site these interactions pull out the top of the active site and consequently change the shape of the active site cleft. In contrast to aspartate, glycine at position 242 in the CTX-M-15 β-lactamase tends to be small in size and hydrophobic. These properties enable glycine to be placed in regions of the protein where other amino acids are unlikely to occur. As a result, glycine will reduce the top of the active site cleft decreasing substrate affinity and relatively increasing the values of MICs to cephalosporins (Table 31).

CTX-M	Strain	MICs of Ceftazidime (ml/L)	MICs of Cefoxitin (ml/L)
CTX-M-15	4, 15, 17, 20, 37, 47, 55, 57, 60, 61, 74, 75, 80, 82, 87, 88, 90, 91, 92, 93, 94, 95, 96, 98, 99, 100, 101, and 105	**32**	**2-128**
CTX-M-3	102	**8**	**8**

Table 31: A comparison of the MIC values of ceftazidime and cefoxitin in strains carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-3}}$.

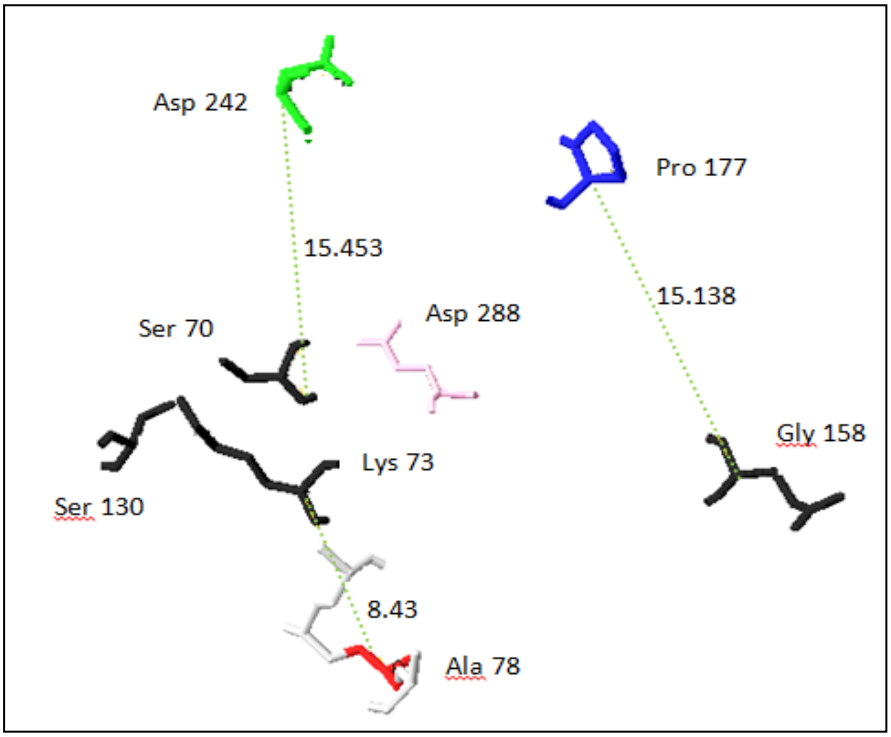


Figure 36: Aspartate residue at position 242 in CTX-M-3. The active site of the enzyme is coloured in black. The structure was viewed in DeepView.

In Figure 37, the molecular structure of CTX-M-28 can be seen. The only difference between CTX-M-15 and CTX-M-28 lies at position 288 and it is asparagine for aspartate. Those two amino acids do not have considerable differences in their properties. Therefore, no effect can be seen on the structure of the protein and no change in the MICs values as well (Table 32).

CTX-M	Strain	MICs of Ceftazidime (ml/L)	MICs of Cefoxitin (ml/L)
CTX-M-15	4, 15, 17, 20, 37, 47, 55, 57, 60, 61, 74, 75, 80, 82, 87, 88, 90, 91, 92, 93, 94, 95, 96, 98, 99, 100, 101, and 105	**32**	**2-128**
CTX-M-28	86	**32**	**8**

Table 32: A comparison of the MIC values of ceftazidime and cefoxitin in strains carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-28}}$.

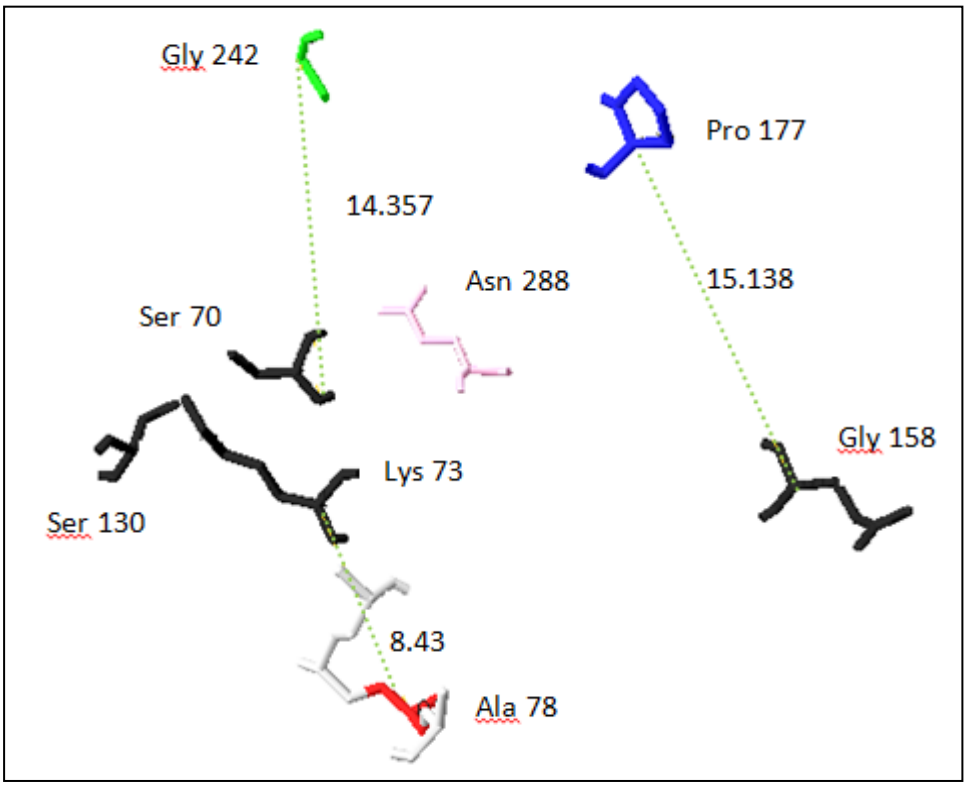


Figure 37: Asparagine residue at position 288 in CTX-M-28. The active site of the enzyme is coloured in black. The structure was viewed in DeepView.

5 Discussion

5.1 Phenotypic and molecular identification of *bla*$_{CTX-M}$ genes

It was important to elucidate the locations and the populations being served in each hospital in Figure 7 to show the most common CTX-M β-lactamase member not only as the most prevalent in the selected hospitals, but also among different populations and from different districts in Kuwait. Also, to describe the situation of nosocomial infections in Kuwait rather than focusing on a certain hospital and handling CTX-M prevalence from one perspective view. Moreover, the elucidation of the Kuwaiti hospitals on Kuwait's map was to detect any possible transmission of the most common CTX-M gene among those hospitals. Nevertheless, the lack of epidemiologic data such as the wards of the patients, the nationality, and the diagnosis made the explanation and the understanding of the association of the prevalence of CTX-M genes in different hospitals difficult to explain.

ESBL identification in Kuwaiti hospitals is usually carried out with Vitek 2 system, DDD method and in some hospitals with E-test strips (Al Hashem *et al.*, 2011, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Dashti *et al.*, 2010a, Dashti *et al.*, 2010b). Importantly, no genotypic identification has been done in any of the aforementioned hospitals. All of the strains were phenotypically identified as ESBL-producers. Nevertheless, CTX-M-production was not identified genotypically in these hospitals. Rather, genotypic identification of CTX-M was carried out in the

Kuwait University laboratories with PCR. Interestingly, all of the strains were identified as CTX-M-producers, indicating the spread of cefotaxime resistance in Kuwaiti hospitals. The increased prevalence of CTX-M enzymes was also reported in other studies from Kuwait (Al Hashem *et al.*, 2011, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Ensor *et al.*, 2009, Rotimi *et al.*, 2008). Moreover, CTX-M enzymes are reported to be the most prevalent family of ESBLs (Naseer *et al.*, 2009). The increased prevalence of this family of enzymes is commonly associated with *E. coli* and *K. pneumoniae* (Dutour *et al.*, 2002, Eckert *et al.*, 2006, Girlich *et al.*, 2009, Naseer *et al.*, 2009, Peirano and Pitout, 2010, Pitout, 2010, Rice, 2012, Yoo *et al.*, 2010).

The findings in this work correlate with previous studies indicating that the prevalence of CTX-M enzymes is mainly associated with UTIs (Woodford *et al.*, 2009). The global description of CTX-M enzymes and the spread of the members of this family of enzymes indicated the presence of ''CTX-M pandemic'' (Canton and Coque, 2006, Ensor *et al.*, 2009, Leflon-Guibout *et al.*, 2004).

From this finding I can report the occurrence of ''CTX-M pandemic'' as well as the need for the screening of CTX-M-production genotypically in hospital settings. This might assess clinicians in directing therapeutic options. Moreover, genotypic detection of CTX-M-production in hospitals might help in providing the infection control unit a proper surveillance of burgeoning antibiotic resistance pattern in nosocomial settings in Kuwait. In addition, genotypic detection of these enzymes might influence the effectiveness of antibiotic restriction policies used in Kuwait.

The association of the production of CTX-M enzymes by Gram-negative bacteria in UTIs was reported from different hospitals around the world (Kanj *et al.*, 2008). UTIs with *E. coli* are thought to be one of the most important hospital-acquired infections. The peculiar feature of *E. coli* is that it causes nosocomial infections with polyclonal features, making it difficult for infection control units to determine the origin and the source of the infection (Oteo *et al.*, 2010). Another important hospital-acquired infection is caused by *K. pneumoniae* (Wang *et al.*, 2009). *K. pneumoniae* is known to cause pneumonia, UTIs, and intra-abdominal infections

(Shi *et al.*, 2009, Bagattini *et al.*, 2006). Therefore, in sample collection I focused on nosocomial infections caused by *E. coli* and *K. pneumoniae* strains. CTX-M-production associated with *E. coli* and *K. pneumoniae* has been increasingly reported worldwide (Dutour *et al.*, 2002, Eckert *et al.*, 2006, Naseer *et al.*, 2009, Woodford *et al.*, 2006). and a similar situation is present in Kuwait, where CTX-M-production is increasingly associated with *E. coli* and *K. pneumoniae*. Not only this study reports such association, but also previous, less comprehensive studies from Kuwait support this finding (Al Hashem *et al.*, 2011, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Ensor *et al.*, 2009, Rotimi *et al.*, 2008).

5.2 Pulsed-field gel electrophoresis

PFGE is considered to be one of the essential and highly discriminatory tools used in the field of molecular biology (Maslow *et al.*, 1993, Singh *et al.*, 2006, Lahti, 1996). It also serves as a tool for sizing and profiling plasmids and chromosomal mapping of different prokaryotes (Maslow *et al.*, 1993). Within hospital laboratories, the use of PFGE is required in monitoring the spread of infections and tracking diseases causing outbreaks in different settings (Bannerman *et al.*, 1995, Barrett *et al.*, 1994, Bohm and Karch, 1992, Gautom, 1997, Lahti, 1996).

In this work, PFGE was performed to detect the source or to track the spread of cefotaxime resistance in some Kuwaiti hospitals. Moreover, PFGE was done to find the clonal relationship among the collected isolates and use a representative strain from each clone for further sequencing, plasmid, and genetic environment studies. The representative strains would have helped in seeing the full picture of cefotaxime resistance in Kuwait with further studies. Unfortunately, poly-clonality was observed. In addition, the results of PFGE seen in Figure 10 and Figure 11, indicated the spread of $bla_{\text{CTX-M}}$ genes by means of HGT mediated by mobile genetic elements like IS*Ecp1*. Few strains were identical and similar (22 strains). Consequently, no representative strain was selected for further studies in this work. Instead, selected isolates were chosen on the basis of their resistance profiles.

Traditional PFGE is inconvenient in terms of time and laboour (Alaidan *et al.*, 2009, Gautom, 1997). PFGE is technically demanding, requiring up to 6 days procedure

from the day of isolating the bacterial strain (Goering and Winters, 1992, Matushek *et al.*, 1996, Miranda *et al.*, 1996). Therefore, a rapid and cost-effective method was developed to overcome these drawbacks. The protocol described in this study was assessed by modifying the method described by Durmaz *et al* (Durmaz *et al.*, 2009) and comparing it to that of Miranda *et al* (Miranda *et al.*, 1996).

5.3 Sequencing with plasmid and genetic environment studies

Upon PCR amplification, *bla*$_{CTX\text{-}M\text{-}15}$ was found to be the most prevalent member of CTX-M family (Table 24). Previous studies from Kuwait reported the presence of *bla*$_{CTX\text{-}M\text{-}15}$ (Al Hashem *et al.*, 2011, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Ensor *et al.*, 2009, Rotimi *et al.*, 2008). The first description of *bla*$_{CTX\text{-}M\text{-}15}$ was reported from India (Karim *et al.*, 2001). Ever since, *bla*$_{CTX\text{-}M\text{-}15}$ has gained a global reputation spreading into different continents (Novais *et al.*, 2007, Pitout, 2010). Moreover, CTX-M-15 gene has been fairly reported in association with *E. coli* and *K. pneumoniae* strains (Coelho *et al.*, 2010, Woodford *et al.*, 2006). Also, previous reports indicated that the most prevalent type of ESBLs is *bla*$_{CTX\text{-}M\text{-}15}$ gene with MDR pattern (Nicolas-Chanoine *et al.*, 2008, Peirano and Pitout, 2010). The results in this study agree with previous studies, confirming that the most prevalent member of ESBLs in Kuwait is *bla*$_{CTX\text{-}M\text{-}15}$ (Coque *et al.*, 2008, Ensor *et al.*, 2009, Dashti *et al.*, 2010b, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011). The sequencing results are suggestive the acquisition of *bla*$_{CTX\text{-}M}$ genes in three different phases and from three different ancestors. Fifteen isolates encoding *bla*$_{CTX\text{-}M\text{-}15}$ belonging to one progenitor (isolate 4, 15, 17, 20, 37, 47, 55, 57, 60, 61, 74, 88, 100, 101, and 105), 12 isolates belonging to different progenitor (isolate 75, 80, 82, 87, 90, 91, 92, 93, 94, 95, 96, and 98), and one isolate (isolate 99) belonging to a different proginator from the previous two. This could be explained by the nucleotide differences described when comparing all of the isolates encoding *bla*$_{CTX\text{-}M\text{-}15}$ as seen in Figure 19. The nucleotide differences found among the reported *bla*$_{CTX\text{-}M\text{-}15}$ genes are indicative of

synonymous mutations. These mutations do not change the amino acid sequences and consequently have no effect on the protein's structure or function. They only point to possible different diversification processes or origins and support the poly-clonality of *E. coli* strains found and the poly-clonality described in the PFGE results (Figure 10). Moreover, when the isolates share the same clone it does not necessarily mean having the same genetic context. This can be seen in isolate 15 and 74 with isolate 80 and 82. The first two isolates proved to be from the same clone and the second two from another. Yet, both sets of isolates share the same genetic context (A) in Figure 30. Also, the same event can be seen in isolate 91 and 92 with isolate 95 and 96, where the first two isolates share the same clone indicated by the nucleotide sequences differences in Figure 19 and the last two coming from a different clone. Regardless, both sets of isolates share the same genetic platform. The synonymous mutations are also indicative of recent *in vivo* mutations being exerted by the antibiotic selective pressure which was hypothesized from the resistance profile of the isolates used in this study. This finding is additionally supported by the presence of $bla_{\text{CTX-M-3}}$ in an earlier collection in 2006 (Table 6). There is one study that indicated that $bla_{\text{CTX-M-3}}$ is the parent enzyme of CTX-M-1 sub-group rather than $bla_{\text{CTX-M-1}}$ (Novais *et al.*, 2010). Therefore, it can be assumed that the reported $bla_{\text{CTX-M-3}}$ is the parental enzyme of some of the CTX-Ms found in this study and the extensive use of cefotaxime and ceftazidime probably acted as a driving force for the diversification of $bla_{\text{CTX-M-3}}$ gene into $bla_{\text{CTX-M-15}}$ gene and further into other CTX-Ms reported in this work.

The spread of *bla*$_{CTX\text{-}M\text{-}15}$ gene is possibly linked to the presence of the insertion sequence, IS*Ecp1*. Strong association between *bla*$_{CTX\text{-}M\text{-}15}$ and IS*Ecp1* has been reported in literature (Ma *et al.*, 2009, Naseer *et al.*, 2009, Poirel *et al.*, 2003, Sonnevend *et al.*, 2006). Moreover, the acquisition of CTX-M genes is mainly mediated by IS*Ecp1* (Yu *et al.*, 2007, Yu *et al.*, 2011, Zheng *et al.*, 2012). Insertion sequences are DNA segments that encode proteins and enzymes required for mediating the movement of adjacent DNA sequences between replicons (Frost *et al.*, 2005). IS*Ecp1*, a member of IS*380* family have been extensively reported in literature to be associated with members of *bla*$_{CTX\text{-}M\text{-}1}$, *bla*$_{CTX\text{-}M\text{-}2}$, *bla*$_{CTX\text{-}M\text{-}9}$, and *bla*$_{CTX\text{-}M\text{-}25}$ families (Smet *et al.*, 2010, Yu *et al.*, 2007, Yu *et al.*, 2011, Zheng *et al.*, 2012). These insertion sequences are responsible for the capture, mobilization, transposition, and expression of different *bla*$_{CTX\text{-}M}$ genes (Sonnevend *et al.*, 2006, Sun *et al.*, 2010, Zong *et al.*, 2010). IS*Ecp1* was present in the upstream sequences of all of the CTX-Ms reported in this work, but with variable distances (Figure 30). Similar observations were reported in previous studies (Dhanji *et al.*, 2011, Kiratisin *et al.*, 2007, Novais *et al.*, 2007). This gene was carried on IncFII plasmid that is known for its narrow host range compatibility but being pandemic and highly associated with the spread resistance genes in *E. coli* strains (Diestra *et al.*, 2009, Peirano and Pitout, 2010). Regarding other isolates with IS*Ecp1* upstream *bla*$_{CTX\text{-}M\text{-}15}$, but without a spacer sequence in between, is similar to previous studies reporting a similar structure (Ma *et al.*, 2011, Sun *et al.*, 2010). Additionally, the size of the IS*Ecp1* being found (400bp) was also the same as those previous studies. These structures are seen in (D) and (E) in Figure 30 (isolate 37, 47, 90, 91, 92, 93, 95, 96,

99, and 100) (Gonullu *et al.*, 2008, Oteo *et al.*, 2006). This study confirms the findings of others by describing various variable distances between IS*Ecp1* and $bla_{\text{CTX-M-15}}$ (Dhanji *et al.*, 2011, Kiratisin *et al.*, 2007, Novais *et al.*, 2007). The variable distances between IS*Ecp1* and the downstream $bla_{\text{CTX-M}}$ genes indicate separate escape events of the $bla_{\text{CTX-M}}$ gene from the origin and the acquisition of genes in different phases (Woodford *et al.*, 2009). This provide further support of the different progenitors of the $bla_{\text{CTX-M-15}}$ genes found as they vary in their distance from the upstream IS*Ecp1* (49bp, 10 bp with a conserved spacer, conserved spacer only, no distance) (Figure 30). In other words, the point of insertion of IS*Ecp1* is different and it is not consistent even in the same gene (i.e. $bla_{\text{CTX-M-15}}$). This finding contradicts that of Ma *et al* (2011), which suggests that the point of insertion of IS*Ecp1* upstream $bla_{\text{CTX-M}}$ gene is dependent on the CTX-M-type. By contrast, this study suggests that there is no preferential point of insertion of IS*Ecp1* for a certain $bla_{\text{CTX-M}}$ gene. One study by Novais (2007) indicated that different locations of IS*Ecp1* insertion upstream $bla_{\text{CTX-M-15}}$ onto different plasmids platforms are suggestive of the occurrence of independent mobilization events but originating from the same ancestor (Novais *et al.*, 2007).

From Figure 30, IS*Ecp1* is the most common factor present in the genetic context of $bla_{\text{CTX-M-15}}$ gene, and it is leading for its mobilization. IS*Ecp1*, also play a role in HGT and evolutionary process of CTX-M family of enzymes (Ensor *et al.*, 2009, Gangoue-Pieboji *et al.*, 2005). In this work, the *tnpA* gene of the Tn*3* transposon is disrupted

by the IS*Ecp1* upstream $bla_{\text{CTX-M-15}}$ gene as seen in structure (B), (C), (D), (E), and (F) in Figure 30. These structures are found in most of the isolates encoding $bla_{\text{CTX-M-15}}$ (isolate 20, 37, 47, 57, 61, 88, 90, 91, 92, 93, 94, 95, 96, 99, and 100) and are indicative of transpositional processes mediated by IS*Ecp1*. These findings agree with a previous study by Smet (2010). Another evidence of transpositional processes mediated by IS*Ecp1*, is the presence of 5 bp target site duplication, that is usually A-T rich (Smet *et al.*, 2010, Woodford *et al.*, 2009). One of the target site duplication is TATGA (Smet *et al.*, 2010). This 5 bp signature of transpositional processes mediated by IS*Ecp1* can be seen in the upstream sequence of $bla_{\text{CTX-M-15}}$ gene in isolates; 20, 47, 75, 80, 82, and 102 (Table 26). Another signature is ''TATTG'' described by Woodford *et al* (2009) is also seen in the upstream sequence of $bla_{\text{CTX-M-15}}$ gene of isolates; 37, 61, and 89 (Table 26). Two different 5 bp sequences were described by Smet (2010) and were also seen in isolate 37, these sequences are ''ATATA'' and ''TTATA'' (Table 26). In isolate 37, the presence of 3 signatures of transpositional processes indicates the occurrence of three transpositional events happening in different timings. Consequently, this may justify further diversification of $bla_{\text{CTX-M-15}}$ gene and the development of CTX-M-15 derivatives such as $bla_{\text{CTX-M-28}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-117}}$.

Further evidence of mobilization is the presence of partial sequences of ORF477 (Zong *et al.*, 2010). This finding is also seen in most of the strains carrying $bla_{\text{CTX-M-15}}$ gene seen in structure (A), (B), (D), and (F) in Figure 30. These partial sequences are

seen in the downstream genetic contexts of *bla*$_{CTX\text{-}M\text{-}15}$ gene of isolates; 15, 20, 37, 47, 57, 61, 74, 75, 80, 82, 86, 91, 92, 95, 96, 99, and 100 (Table 26). These sequences are suggestive of a preliminary capture of *bla*$_{CTX\text{-}M\text{-}15}$ gene followed by a mobilization process and a transpositional event mediated by IS*Ecp1* (Sun *et al.*, 2010, Zong *et al.*, 2010). These pieces of evidence of transposition signatures and remnants of sequences further point to the mobilization, transpositional events, and diversification of *bla*$_{CTX\text{-}M\text{-}15}$ among the collected isolates (Canton *et al.*, 2012, Coelho *et al.*, 2010, Lartigue *et al.*, 2004).

The role of IS*Ecp1* is not only restricted to capturing and mobilizing *bla*$_{CTX\text{-}M\text{-}15}$; it also acts in increasing the expression of the downstream *bla*$_{CTX\text{-}M\text{-}15}$ genes (Dutour *et al.*, 2002, Eckert *et al.*, 2006, Ma *et al.*, 2011, Peirano and Pitout, 2010). One study by Ma (2011) suggested that IS*Ecp1* adjacent to the *bla*$_{CTX\text{-}M\text{-}15}$ gene and with shorter distances to that *bla*$_{CTX\text{-}M\text{-}15}$ gene are necessary for the maintenance of high level expression of that *bla*$_{CTX\text{-}M\text{-}15}$ gene (Ma *et al.*, 2011). A –35 and –10 promoter sequence described by Poirel (2003) ''TTGACA'' and ''TAAACT'' was also found in the upstream sequences of *bla*$_{CTX\text{-}M\text{-}15}$ of isolate; 20, 37, and 40 (Table 26). Another promoter sequence described by Lartigue (2004) ''TGCAG'' was found in the upstream sequence of *bla*$_{CTX\text{-}M\text{-}15}$ of isolates; 20, 47, and 61. Upon analysis, the closest promoter sequences found in the upstream genetic contexts of *bla*$_{CTX\text{-}M\text{-}15}$ were found to be residing in the ''W'' sequences. Additionally, the promoter sequence described by Dhanji (2011) ''TTCATG'' was found in the ''W'' region of all

strains with structures containing this region. These isolates are; 15, 37, 57, 61, 74, 75, 80, 82, 94, and 100 (Table 26) and are seen in structure (A), (F), and (H) in Figure 30. Most importantly, no promoter sequences were found in the conserved spacers described in isolate 20 and 88 that is structure (B) and (C) respectively in Figure 30. The function of the conserved spacer and possible explanation of its existence upstream *bla*$_{CTX\text{-}M\text{-}15}$ remains ambiguous. Further investigation of the ''W'' region was approached in this work. The analysis of the ''W'' region showed that it did not only carry promoters required for the expression of the downstream *bla*$_{CTX\text{-}M}$ genes. Also, it had the Shine-Dalgarno ribosomal binding sequence ''AAGGAA'' adjacent to the start codon of the downstream *bla*$_{CTX\text{-}M}$ gene (Shine and Dalgarno, 1974). The function of this sequence is involved in protein translation. It recruits ribosomes to mRNA for the initiation of protein synthesis by aligning it with the start codon (ATG) of the downstream gene. This sequence was found to be in all of the ''W'' sequences upstream the reported *bla*$_{CTX\text{-}M}$ genes reported in this project.

To answer more questions of the specificity of the ''W'' region to *bla*$_{CTX\text{-}M\text{-}15}$ gene than other CTX-M family members present in this study (like *bla*$_{CTX\text{-}M\text{-}55}$ gene), all of the ''W'' sequences found upstream of the CTX-M genes in this work were taken and aligned with the ''W'' region of *bla*$_{CTX\text{-}M\text{-}55}$ published from Thailand (accession number DQ885477) using the multi align web interface. The results indicated that all of the ''W'' sequences present upstream *bla*$_{CTX\text{-}M\text{-}15}$ are identical to the ''W'' region present upstream *bla*$_{CTX\text{-}M\text{-}55}$ reported in this work and from a previous study

as well (Kiratisin *et al.*, 2007) (Figure 38). Only on nucleotide difference was present in the ''W'' sequence upstream $bla_{CTX\text{-}M\text{-}15}$. Mutations in the Shine-Dalgarno ribosomal sequence result in reducing the translation process of the downstream gene, and therefore reducing the expression of that gene. This can be noticed from the MIC of ceftazidime of isolate 89 carrying the upstream environment of GenBank accession number FJ235691.1. Therefore it can be presumed that this is responsible for the lower MIC of ceftazidime than that found in CTX-M-15 β-lactamase-producing strain. Instead, the levels of MICs of ceftazidime in this strain are higher than those in CTX-M-15. This could be explained by the mutational effect in $bla_{CTX\text{-}M\text{-}15}$ that led to certain changes in the biochemical properties of the enzyme's active site and consequently ncreasing the binding of the enzyme's active site by ceftazidime. Yet, another explanation of the action of the mutational in the Shine-Dalgrano ribosomal sequence is the lower prevalence of this enzyme than $bla_{CTX\text{-}M\text{-}15}$. Eventually, $bla_{CTX\text{-}M\text{-}55}$ gene is not expressed as efficient as $bla_{CTX\text{-}M\text{-}15}$ gene.

Nevertheless, the alignment indicated that the sequence of the ''W'' region could be variable, in terms of few nucleotide changes, even in the same $bla_{CTX\text{-}M}$ member and there is no specific sequence of this region for a specific $bla_{CTX\text{-}M}$ member.

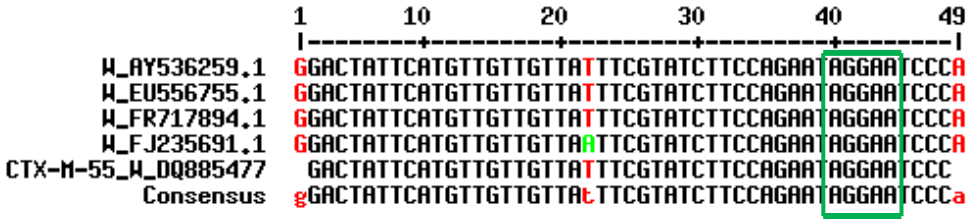


Figure 38: Alignment of the ''W'' region present upstream *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{CTX\text{-}M\text{-}55}$. Nucleotides in black colour are with high consensus, red with low consensus, and green with neutral consensus. Shine-Dalgrano is highlighted in the green box.

Presumably, the promoter sequences found in the ''W'' regions act in increasing the expression of the downstream *bla*$_{CTX\text{-}M\text{-}15}$ gene as suggested earlier. This could justify the high level of resistance to cefotaxime in the collected isolates. Additionally, this could further explain the presence of the ''W'' region upstream *bla*$_{CTX\text{-}M\text{-}15}$ gene. It is noteworthy, that the presence of the ''W'' region, though it is important for the expression of *bla*$_{CTX\text{-}M\text{-}15}$ gene, is not conditional for the existence of *bla*$_{CTX\text{-}M\text{-}15}$ gene as seen in structure (D) and (E) in Figure 30. Moreover, the IS*Ecp1* could be present upstream *bla*$_{CTX\text{-}M\text{-}15}$ gene without the presence of the ''W'' sequence seen in structure (B), (C), (D), and (E) in Figure 30. Above all, the ''W'' region is not specific to a certain strain carrying *bla*$_{CTX\text{-}M\text{-}15}$ gene, as it is reported in this work to be upstream *bla*$_{CTX\text{-}M\text{-}15}$ gene encoded by *E. coli* and *K. pneumoniae* in isolate 15 and 80 respectively (Table 26). The presence of the ''W'' region was also observed upstream *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{CTX\text{-}M\text{-}3}$ genes described in French, India, and Turkey (Lartigue *et al.*, 2003).

From the sequencing results and from the analysis of the IS*Ecp1* provided herein, I can confirm and agree with previous studies that IS*Ecp1* is a key factor in the increased spread and expression of $bla_{\text{CTX-M-15}}$ gene. Admittedly, IS*Ecp1* is the main cause of the widespread of CTX-M-15 enzyme in Kuwaiti hospitals along with the extensive use of cefotaxime and ceftazidime acting as the driving force.

This is the first work from Kuwait describing the genetic conformation of Kuwaiti isolates carrying $bla_{\text{CTX-M-15}}$ gene. Nevertheless, previous studies indicated the presence of IS*Ecp1* and IS*26* upstream $bla_{\text{CTX-M-15}}$, but did not elucidate further genetic sequences and orientation upstream and downstream $bla_{\text{CTX-M-15}}$ gene (Al Hashem *et al.*, 2011, Al Sweih *et al.*, 2010, Al Sweih *et al.*, 2011, Coque *et al.*, 2008, Dashti *et al.*, 2010b, Ensor *et al.*, 2009, Rotimi *et al.*, 2008).

From the genetic contexts of $bla_{\text{CTX-M-15}}$ sought in this work, variability is well observed and no consistent genetic platform for $bla_{\text{CTX-M-15}}$ gene was documented. In fact, five different genetic environments for $bla_{\text{CTX-M-15}}$ gene were represented in structure (A), (B), (D), (F), (G), and (H) (Figure 30). Not only has this work showed the variability of the genetic environments of $bla_{\text{CTX-M-15}}$ gene, but also other studies indicated comparable findings though in different countries (Dhanji *et al.*, 2011, Oteo *et al.*, 2006). The most common genetic context of $bla_{\text{CTX-M-15}}$ gene in *E. coli* strains was represented in structure (D) (Figure 30). This genetic arrangement was observed in eight strains (isolate 37, 47, 91, 92, 95, 96, 99, and 100). Notably, this

structure was not observed in any of the collected *K. pneumoniae* strains encoding *bla*$_{CTX\text{-}M\text{-}15}$ gene. Furthermore, this genetic platform was previously reported in a study from Haiti in *A. baumannii* strain carrying *bla*$_{CTX\text{-}M\text{-}15}$ gene (Potron *et al.*, 2011). Features of this structure are; the presence of a truncated IS*Ecp1* sequence of 339 bp long linked directly to *bla*$_{CTX\text{-}M\text{-}15}$ gene. The direction of the IS*Ecp1* upstream is opposite to the downstream *bla*$_{CTX\text{-}M\text{-}15}$ gene. The inverse direction of IS*Ecp1* extend conclusion that isolates sharing this genetic structure have acquired *bla*$_{CTX\text{-}M\text{-}15}$ gene in separate escape events than isolates with IS*Ecp1* in the same direction as the downstream *bla*$_{CTX\text{-}M\text{-}15}$ gene. A previous study related the inverse direction of the IS*Ecp1* upstream the *bla*$_{CTX\text{-}M}$ gene to different mobilization events (Smet *et al.*, 2010). The plasmid sizes carrying *bla*$_{CTX\text{-}M\text{-}15}$ gene with this genetic context ranged from 60-130 Kb and most of them were not typed into a certain incompatibility group. However, I maintained to determine the incompatibility grouping of two isolates and both belonged to InFII. In fact, IncFII are known for being narrow-host range plasmids (del Solar *et al.*, 1996). Also, it has been described that IncFII plasmids have conjugative properties (Coelho *et al.*, 2010). The presence of *bla*$_{CTX\text{-}M\text{-}15}$ gene has been noted to be associated with IncFII plasmids in different reports and this work agree with these studies (Canton *et al.*, 2012, Golebiewski *et al.*, 2007, Lavollay *et al.*, 2006, Naseer *et al.*, 2009, Peirano and Pitout, 2010). Probably, the inability to detect the incompatibility grouping of other plasmids in this study was due the complexity of these plasmids. The complexity of plasmids increases with their sizes, in which they might contain, co-integrated compatible replicons. Therefore, the inability to measure plasmid competition might be justified by failure

of designing appropriate molecular probes to detect these co-integrated compatible replicons (Frost *et al.*, 2005).

The second most common genetic arrangement of *bla*$_{CTX\text{-}M\text{-}15}$ found was represented in structure (A) (Figure 30). This genetic arrangement was described in six isolates encoding *bla*$_{CTX\text{-}M\text{-}15}$ gene. Moreover, this genetic context was also previously reported to be ''the international *bla*$_{CTX\text{-}M\text{-}15}$ genetic environment'' (Diestra *et al.*, 2009). The international genetic environment of *bla*$_{CTX\text{-}M\text{-}15}$ gene was not specific for *E. coli* strains only (isolate 15, 74, 75, and 86). Instead, it was also found in two *K. pneumoniae* strains (isolate 80 and 82). Consequently, structure (A) in Figure 30 is common to different bacterial strains carrying *bla*$_{CTX\text{-}M\text{-}15}$ gene. It is noteworthy that the latter genetic arrangement was not described in other genes belonging to *bla*$_{CTX\text{-}M\text{-}1}$ group. Accordingly, this showed the uniqueness of this genetic context to the *bla*$_{CTX\text{-}M\text{-}15}$ gene. In other words, the international genetic environment of *bla*$_{CTX\text{-}M\text{-}15}$ is not common to *bla*$_{CTX\text{-}M\text{-}3}$ (a possible parent of *bla*$_{CTX\text{-}M\text{-}15}$), which suggests the occurrence of separate transpositional and escape events far from *bla*$_{CTX\text{-}M\text{-}3}$ gene were the source of *bla*$_{CTX\text{-}M\text{-}15}$. Common elements of the international genetic environment of *bla*$_{CTX\text{-}M\text{-}15}$ gene are; the ''W'' region, a disrupted IS*Ecp1* sequence with promoters in that sequence increasing the expression of the downstream *bla*$_{CTX\text{-}M\text{-}15}$ gene, and the presence of ORF 477 downstream the *bla*$_{CTX\text{-}M\text{-}15}$ (Dhanji *et al.*, 2011). In this work, the downstream genetic environment of all the reported CTX-Ms was found to be a hypothetical protein of variable lengths (430-470 bp) encoding ORF 477 (Figure 30). It was not possible to determine the downstream genetic environment in isolates 88, 90, and

93. The last two isolates (90 and 93) are thought to carry chromosomally-encoded *bla*$_{CTX\text{-}M\text{-}15}$ genes with the absence PCR amplicons upon amplification of the plasmids DNA bands with primers described by Dutour (2002).

The genetic environments sought for isolate 20 and 88 are the same, except in the downstream genetic environment of isolate 88 which was not determined as seen in structure (B) and (C) respectively in Figure 30. A similar genetic context was described in *Ent. aerogenes* encoding *bla*$_{CTX\text{-}M\text{-}15}$ gene from Russia (Table 26). Moreover, the same finding was described by Diestra (2009) in an *E. coli* strain encoding *bla*$_{CTX\text{-}M\text{-}3}$ gene from Spain. Both findings show the presence of a truncated IS*Ecp1* upstream *bla*$_{CTX\text{-}M\text{-}15}$ gene and a linking sequence consisting of a 10bp sequence with a conserved spacer of 47bp in length. The role of spacer sequence could not be determined, but one study by Ma (2011) indicated that the spacer sequences upstream the *bla*$_{CTX\text{-}M}$ gene have promoters helping to increase the expression of the downstream *bla*$_{CTX\text{-}M}$ genes. The shorter the sequence, the closer to the *bla* gene and the enhanced activity towards cephalosporins is expressed (Ma *et al.*, 2011). Additional analysis of the spacer sequence indicated the presence of the Shine-Dalgarno ribosomal sequence in the 10 bp near the 5' prime terminus of the start codon of the *bla*$_{CTX\text{-}M\text{-}15}$ gene. As indicated earlier that this sequence function in the translation of the downstream gene.

Two *E. coli* isolates (isolate 57 and 61) encoding *bla*$_{CTX\text{-}M\text{-}15}$ gene share a genetic confirmation reported by Dhanji (2011) to be related to travellers coming from India and Pakistan to the United Kingdom.

As seen in structure (F) in Figure 30, same elements found in the international genetic environment of *bla*$_{CTX\text{-}M\text{-}15}$ gene are found in this genetic context but with additional presence of a truncated IS*26* upstream IS*Ecp1*. This genetic arrangement was also reported in a *K. pneumoniae* strain encoding *bla*$_{CTX\text{-}M\text{-}15}$ gene in Hungary. The presence of IS*26* was also reported to be associated with *bla*$_{CTX\text{-}M\text{-}15}$ gene, but less commonly than IS*Ecp1* (Montesinos *et al.*, 2010). In fact, IS*26* was found to be present upstream *bla*$_{CTX\text{-}M\text{-}15}$ gene on IncFII plasmids with variable sizes described in different reports from Asia and Europe (Novais *et al.*, 2007). This study confirms with an earlier study from Kuwait by Ensor (2009), the association of the presence of *bla*$_{CTX\text{-}M\text{-}15}$ gene with IS*26*. This work also suggests and confirms that IS*26* is not necessarily required for the widespread of *bla*$_{CTX\text{-}M\text{-}15}$ gene (Ensor *et al.*, 2009). Rather, IS*26* was thought to mobilize CTX-M-15 gene by means of homologous recombination than transposition. Additionally, IS*26* was thought to facilitate mobilization of chromosomal fragments as well as DNA from other plasmids and consequently playing a major role in plasmid plasticity and acquiring different AR genes. This could be explain the MDR pattern of isolates 57 and 61 carrying IS*26* that might be due to the presence of AR genes other than *bla*$_{CTX\text{-}M\text{-}15}$ gene mobilized by the present IS*26* (Smet *et al.*, 2010).

The last genetic confirmation described in this work for *bla*$_{CTX\text{-}M\text{-}15}$ gene is seen in (H) in Figure 30 is the same as the most common genetic context of *bla*$_{CTX\text{-}M\text{-}15}$ gene but with a smaller IS*Ecp1* size. This genetic arrangement was observed in *E. coli* isolate (isolate 94). It was also reported to be in *E. coli* strain encoding *bla*$_{CTX\text{-}M\text{-}15}$ in Tunisia (Rejiba *et al.*, 2011).

The results of this study also describe the first report of *bla*$_{CTX\text{-}M\text{-}28}$, *bla*$_{CTX\text{-}M\text{-}55}$, and *bla*$_{CTX\text{-}M\text{-}117}$ in Kuwait and in the Middle East. The CTX-M-3 β-lactamase found in one of the collected strains (strain 102) gene was previously described in the Middle East in Turkey (Acikgoz *et al.*, 2008, Agin *et al.*, 2011, Bahar *et al.*, 2006, Galimand *et al.*, 2005, Nazik *et al.*, 2008, Samuelsen *et al.*, 2009).

Sporadic reports have been describing the presence of *bla*$_{CTX\text{-}M\text{-}28}$ gene, one report from Netherlands (Hasman *et al.*, 2005), two reports from China (Tian *et al.*, 2012, Yu *et al.*, 2007), one report from India (Kingsley and Verghese, 2008), one from Tunisia (Ben Achour *et al.*, 2009), one from France (Dubois *et al.*, 2010), one from Brazil (Lopes *et al.*, 2010), and one from Korea (Tian *et al.*, 2012). The first report of *bla*$_{CTX\text{-}M\text{-}28}$ was documented in 2005 in *Salmonella* spp isolated in the Netherlands. It was indicated that *bla*$_{CTX\text{-}M\text{-}28}$ was present on conjugative plasmids and in animal reservoirs (Hasman *et al.*, 2005). Later, in 2007, another report of *bla*$_{CTX\text{-}M\text{-}28}$ was

documented from China, describing the presence of this gene in *E. coli* and *K. pneumoniae* strains on conjugative plasmids as well. Additionally, the presence of IS*Ecp1* upstream $bla_{\text{CTX-M-28}}$ was also mentioned (Yu *et al.*, 2007). This finding agrees with our results in describing the presence of $bla_{\text{CTX-M-28}}$ in *E. coli* strain on a conjugative plasmid (Table 27). Interestingly, this strain was collected in the same year of the first report of $bla_{\text{CTX-M-28}}$ (i.e. 2007), but identified later. This finding indicates a concomitant appearance of the same gene in two different countries. Therefore, the assumption that $bla_{\text{CTX-M-28}}$ gene was present in this study due to possible immigration from China could be disregarded. Rather, the development of $bla_{\text{CTX-M-28}}$ could be due to the use of ceftazidime acting as a selective pressure on $bla_{\text{CTX-M-15}}$ and driving mutations in a certain direction leading to the evolution of $bla_{\text{CTX-M-28}}$. Also, un-predicted transpositional events were acquired by the presence of the disrupted IS*Ecp1* (i.e. 338 bp long) upstream $bla_{\text{CTX-M-28}}$ gene. The presence of IS*Ecp1* might drive the evolution of $bla_{\text{CTX-M-15}}$ gene into $bla_{\text{CTX-M-28}}$ gene. Evidence of transpositional processes is the presence of a 5bp repeat in the insertion sequence IS*Ecp1* present upstream $bla_{\text{CTX-M-28}}$ (Gangoue-Pieboji *et al.*, 2005). Another support of the hypothesis that $bla_{\text{CTX-M-28}}$ originated from $bla_{\text{CTX-M-15}}$ by transposition is the similar genetic context of $bla_{\text{CTX-M-28}}$ with $bla_{\text{CTX-M-15}}$ encoded by strains collected from the same hospital (IDH) in the same year (strain 75, 80, and 82) (Figure 30). Yet, the cause of diversification and the mutation of $bla_{\text{CTX-M-15}}$ into $bla_{\text{CTX-M-28}}$ around amino acid position 288 cannot be explained, especially when previous studies indicated mutational hot-spots in CTX-M family members. These studies did not describe amino acid position 288 as being one of the mutational hot-spots

whereby mutational events are more likely to occur and develop. These mutational hot-spots are; Asn104, Asn132, Ser237, and Asp240 (Gniadkowski, 2008, Perez *et al.*, 2007). Nevertheless, the position 288 in CTX-M-1 group family members was documented recently to be lying under positive selection pressure (Novais *et al.*, 2010). This probably explains the mutational event occurring at amino acid positions 288 leading to the diversification of $bla_{\text{CTX-M-15}}$ into $bla_{\text{CTX-M-28}}$. Moreover the driving force of the extensive use of ceftazidime promoted the mutation of $bla_{\text{CTX-M-15}}$ into $bla_{\text{CTX-M-28}}$ (Table 25). It is noteworthy that the genetic environment of $bla_{\text{CTX-M-28}}$ has not been previously been reported in the literature and this work describes novel report of the genetic context of this gene. Moreover, the presence of $bla_{\text{CTX-M-28}}$ in the Middle East and specifically in Kuwait is also novel in Kuwait and has not been described earlier.

This work also describes the first description of $bla_{\text{CTX-M-55}}$ gene in the Middle East area and thus in Kuwait. Most reports of $bla_{\text{CTX-M-55}}$ gene have been from Asia (China, Japan, Korea, and Thailand) (Harada *et al.*, 2012, Kiratisin *et al.*, 2007, Li *et al.*, 2010, Ma *et al.*, 2012, Shi *et al.*, 2009, Sun *et al.*, 2010, Tamang *et al.*, 2012, Tian *et al.*, 2012, Yu *et al.*, 2011, Zhang *et al.*, 2011, Zheng *et al.*, 2012). One report was documented from the USA and England (Sjolund-Karlsson *et al.*, 2011, Snow *et al.*, 2012). All reports of $bla_{\text{CTX-M-55}}$ gene were from Enterobacteriaceaeae and mainly from *E. coli*. Importantly, $bla_{\text{CTX-M-55}}$ gene proved to be carried in animals and pets acting as reservoirs for this enzyme (Harada *et al.*, 2012, Li *et al.*, 2010, Ma *et al.*,

2012, Snow *et al.*, 2012, Sun *et al.*, 2010, Tamang *et al.*, 2012, Zheng *et al.*, 2012). Additionally, CTX-M-55 was reported to be located on conjugative plasmids, some of which could not be typed and others were of incompatibility group FII, 70 kb in size (Kiratisin *et al.*, 2007, Ma *et al.*, 2012, Sjolund-Karlsson *et al.*, 2011, Sun *et al.*, 2010, Tamang *et al.*, 2012, Yu *et al.*, 2011, Zheng *et al.*, 2012). These findings agree with results described in this project, as the *bla*$_{\text{CTX-M-55}}$ gene was found to be located on conjugative plasmids that could not be typed by conventional incompatibility grouping protocols and sized 133 kb (isolate 89). Only three reports described the upstream genetic conformation of *bla*$_{\text{CTX-M-55}}$ gene, one of which reported the downstream genetic arrangement as well. This work shows the first description of IS*26* as a part of the upstream region of *bla*$_{\text{CTX-M-55}}$ gene structure (G) in Figure 30. Here, I report the presence of a truncated IS*Ecp1* that is 49 bp upstream of the start codon of *bla*$_{\text{CTX-M-55}}$ gene as seen in structure (G) in Figure 30. This findings correlate with other studies from Thailand and China (Kiratisin *et al.*, 2007, Zheng *et al.*, 2012). In addition, sequence analysis revealed the presence of unusual *tnpA* gene as part of IS*26* rather than IS*Ecp1* identified by Kiratisin *et al* (Kiratisin *et al.*, 2007). The presence of two insertion sequences as well as a transposon gene indicates the action of different and separate mobilization events and recent mutational processes leading to the emergence of *bla*$_{\text{CTX-M-55}}$. Interestingly, intervening promoter sequences were not seen upstream at the 5' terminus of *bla*$_{\text{CTX-M-55}}$, which are usually known to control the expression of the *bla*$_{\text{CTX-M}}$ genes. Another indication of acquiring *bla*$_{\text{CTX-M-55}}$ threw different transpositional events is the direction of the IS*Ecp1* that is opposite to the translation direction of the

downstream *bla*$_{CTX\text{-}M\text{-}55}$ gene as represented in structure (G) in Figure 30. The downstream genetic context of *bla*$_{CTX\text{-}M\text{-}55}$ was seen a hypothetical protein of 462 bp in length encoding ORF477. This was previously indicated in a study from China of *bla*$_{CTX\text{-}M\text{-}55}$ found in *E. coli* (Zheng *et al.*, 2012). The same genetic context of *bla*$_{CTX\text{-}M\text{-}55}$ was found in two isolates (57 and 61) in this work, but with different orientation of the IS*Ecp1*, which was in the same direction of the downstream *bla*$_{CTX\text{-}M\text{-}15}$. This provided more evidence of the hypothesis of the diversification of *bla*$_{CTX\text{-}M\text{-}15}$ into *bla*$_{CTX\text{-}M\text{-}55}$ by different and separate transpositions.

Regarding *bla*$_{CTX\text{-}M\text{-}117}$ gene, this work also describes the first report of this gene in the Middle East and thus in Kuwait. It is also considered to be only the second report of this gene in world. The first report of *bla*$_{CTX\text{-}M\text{-}117}$ was by Geser *et al* (Geser *et al.*, 2012). The later report only indicated the presence of *bla*$_{CTX\text{-}M\text{-}117}$ in animals, but it did not show any information regarding the genetic environment of this gene. Regardless, this project elucidated the genetic conformation of *bla*$_{CTX\text{-}M\text{-}117}$ gene represented in structure (I) in Figure 30. The absence of the ''W'' sequence was noted with only the presence of a truncated IS*Ecp1* of 339 bp. This genetic context upstream the gene was the same as one described upstream *bla*$_{CTX\text{-}M\text{-}15}$ gene in *A. baumannii* strain in Haiti (Potron *et al.*, 2011). The same genetic environment was also present upstream other strains of this work and it was the most commonly found upstream genetic environment of *bla*$_{CTX\text{-}M\text{-}15}$ gene (isolate 37, 47, 91, 92, 95, 96, 99, and 100) represented in structure (D) in Figure 30. Moreover, *bla*$_{CTX\text{-}M\text{-}117}$

gene was located on a conjugative plasmid of 130 kb (Table 27). The incompatibility group of this group could not be detected.

The last genetic context of *bla*$_{CTX-M}$ gene to be discussed in this work is that of *bla*$_{CTX-M-3}$. The genetic context seen in structure (J) in Figure 30, is noted to be unique to *bla*$_{CTX-M-3}$ in this work. Other structures in this project of *bla*$_{CTX-M-15}$ found to have a conserved spacer sequence also had a 10 bp sequence downstream the conserved spacer rather than a conserved spacer only as seen in structures (B) and (C) in Figure 30. The size of the conserved spacer is crucial for the expression levels of the downstream *bla*$_{CTX-M}$ gene. Moreover, the shorter distance of the conserved spacer sequence, the higher the level of expression of the downstream *bla*$_{CTX-M}$ gene. Nevertheless, one study proved that the promoters found within the IS*Ecp1* are more important than those found in the spacer sequence (Ma *et al.*, 2011). The Shine-Dalgarno ribosomal sequence ''AAGGAA'' was also found upstream the *bla*$_{CTX-M}$ gene. All of the Shine-Dalgarno sequences found in this work were the same regardless of the bacterial species (*E. coli* or *K. pneumoniae*).

The reported *bla*$_{CTX-M-3}$ gene was located on a conjugative plasmid of 194 kb in size and could not be typed by conventional incompatibility grouping probes.

5.4 Examination of the structural models of the CTX-Ms found

Upon mutational effects analysis, point mutations of the reported *bla*$_{CTX-M}$ enzymes (*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-28}$, *bla*$_{CTX-M-55}$, and *bla*$_{CTX-M-117}$) were clustered in five areas nearby the active site of the ESBL enzymes. These amino acid positions were; 80, 177, 242, and 282 (Table 25).

The consensus pattern of the active site of Ambler class A ESBLs is [FY]-x-[LIVMFY]-{E}-S-[TV]-x-K-x(3)-{T}-[AGLM]-{D}-{KA}-[LC]. In CTX-Ms, the active site (S-K-S-E) was found at amino acid positions 70, 73, 130, and 158. The amino acid changes were near the active site of the reported enzymes and in some manner altered the shape of the active site indirectly. Some changes reduced the binding of β-lactamase to cephalosporins such as the changes in *bla*$_{CTX-M-117}$. Other changes increased the binding of β-lactamase to cephalosporins such as the changes found in *bla*$_{CTX-M-15}$ and in *bla*$_{CTX-M-55}$. Other changes did not affect the active site's shape and consequently did not exert any effect on the enzyme's function which could be seen in changes occurring in *bla*$_{CTX-M-28}$. The data suggesting the influence of specific amino acid changes effects are poor. Moreover, the absence of valuable kinetic studies of CTX-M-1 group of enzymes with amino acid changes and rate of spectrum was noticed. However, one study by Novais *et al* (Novais *et al.*, 2010), indicated areas under positive selection of CTX-M-1 group of enzymes with spectrum of activity towards cephalosporins. The amino acid positions being under positive

selection were; 77, 114, 167, 240, and 288. In the latter study, point mutations at those positions produced successful mutants with increased ceftazidime and/or cefotaxime activity. Ceftazidime was identified as a selective pressure and the driving force for the production of these mutations.

Accordingly, and based on the biochemical properties of the amino acid mutations along with the resistance profiles that were reported for *bla*$_{CTX-M}$ enzymes, this work showed that ceftazidime hydrolysis pattern changed in relation to mutations and amino acid changes.

The structure-function relationship was studied in the light of the values of the MICs of cefoxitin and ceftazidime. Also, the identification of the properties of amino acids changes helped in predicting the effect on the active site structure and the function of the enzyme. This method used in this project proved to be useful for the construction of the structural models of the CTX-M enzymes reported in this work.

It is probable that more change would have been seen if there were available crystal structures of any of the CTX-M-1 group of enzymes. Therefore I used the crystal structure for CTX-M-9 enzyme published by Nichols *et al* (2012). The CTX-M models were constructed by the sequences of these enzymes with the use of CTX-M-9 as a backbone. This approach made it possible to visualize the structural

mutations and it was useful for defining different amino acid changes and proximity to the active site. Nevertheless, the method could not detect major changes to the proteins' backbone that might be due to differences between CTX-M-9 and CTX-M-1 group enzymes reported in this study. Regardless, structural changes were detected at positions 80, 177, 242, and 282.

The examination of the structural models allowed the explanation and confirmation of the effect of different amino acid changes on the active site and hence the function of the CTX-M enzyme described. Importantly, amino acid changes occurring at position 80, 177, and 242 occurring in CTX-M-55, CTX-M-117, and CTX-M-15 respectively change the hydrolytic profile of these enzyems to ceftazidime and cefoxitin.

When the amino acid changed in relation to the occurrence of the mutation, the properties of the amino acids changed as well. The different properties of the later amino acids indirectly exerted an effect on the structure of the active site. The amino acid change closest to the active site with different biochemical properties affected the structure of the active site and as a result the function of the active site in binding to β-lactam cephalosporins. Changes of the biochemical properties of the different amino acids at positions where mutations occurred in the reported CTX-M enzymes are explained in Figure 39.

The amino acid change at position 80 from Alanine to Valine increased the hydrolytic activity towards cephalosporins. The MIC levels were higher of ceftazidime and cefoxitin in isolates carrying CTX-M-55 than those carrying CTX-M-15 (Table 29). The increased hydrolytic activity towards ceftazidime was also reported in other studies (Harada *et al.*, 2012, Kiratisin *et al.*, 2007, Ma *et al.*, 2012, Tamang *et al.*, 2012, Yu *et al.*, 2011, Zheng *et al.*, 2012). On the other hand, decreased activity towards cephalosporins seen in amino acid changes occurring at position 177 (Proline→glutamine) in CTX-M-117 was reported in this study (Table 30) and in one other study (Geser *et al.*, 2012).

Changes occurring at amino acid position 242 (Aspartate→Glycine) in CTX-M-15, CTX-M-28, CTX-M-55, and CTX-M-117 will increase the hydrolytic profile of the enzymes with this mutation towards cephalosporins. The increase in the MIC of ceftazidime and cefoxitin can be seen when comparing the MICs of the isolate carrying $bla_{\text{CTX-M-3}}$ gene and other isolates with $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-28}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-117}}$ (Table 29 to Table 32).

The mutation at position 282 (Aspartate→asparagine) seen in CTX-M-28 seemed to exert no effect on the MICs to ceftazidime or cefoxitin (Table 32).

All of the mutational changes and their amino acid positions are simplified in Figure 39.

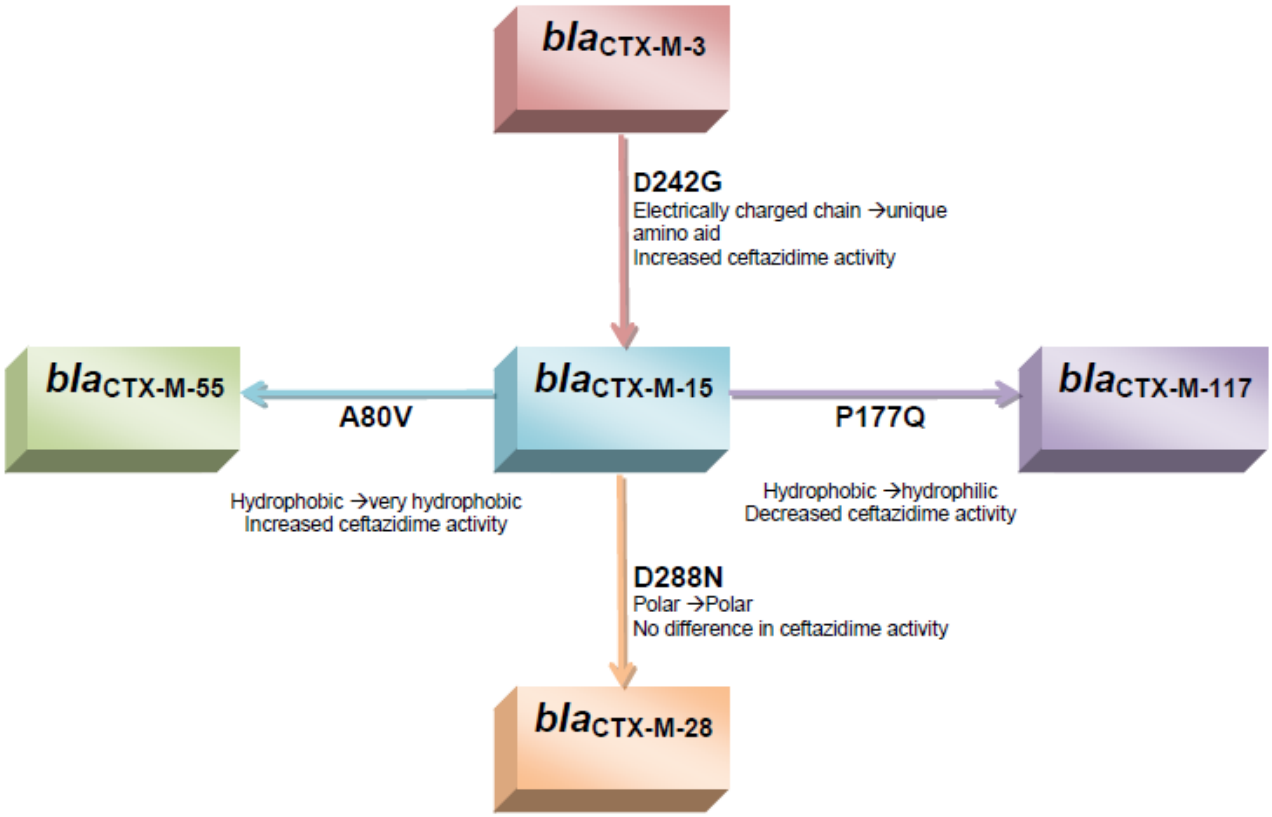


Figure 39: CTX-M reported in this study with mutations and hydrolytic activity towards ceftazidime. A indicates Alanine, D indicates Aspartate, G indicates glycine, N indicates asparagine, P indicates proline, and Q indicates glutamine.

6 Conclusion

In this work, the detection of cefotaximases was done by two means; phenotypic and genotypic. The screening of cefotaxime-resistance was carried out by Vitek 2 system as well as DDD method with cefotaxime and clavulanic acid. The spread of cefotaxime resistance in Kuwaiti hospitals was found to be worrisome. For further detection and conformation of the ESBL-enzyme producing-cefotaximases, PCR was implemented. The results showed that CTX-M-ESBL-producers were responsible for cefotaxime resistance in all of the collected *E. coli* and *K. pneumoniae*.

To fulfil the second objective of this project, the MICs were determined using four different classes of antibiotics. The determination of MIC was carried out namely by the double agar dilution method described by BSAC. Interestingly, co-resistance to flouroquinolones and aminoglycosides was found to be significantly increased in both strains. Additionally, resistance to other generations of cephalosporins was also reported. Surprisingly, all of the collected *E. coli* and *K. pneumoniae* were found to be susceptible to carbapenems.

The spread of CTX-M-producers led to the need of the detection of possible relatedness among the collected isolates. Therefore, PFGE was maintained to study the clonality of the study isolates. The original PFGE method described by Miranda e*t al* (1996) was thought to be time-consuming and demanding for typing a large number of isolates. Therefore, an enhanced PFGE method was developed and used to type the study isolates. The enhanced PFGE method could be described as rapid,

producible, time and cost-effective method that can be used for epidemiologic studies. Nevertheless, the results of PFGE indicated poly-clonality of the collected isolates. Hence, indicating the spread of CTX-ESBL enzymes by HGT mediated by MGE.

Upon further amplifications, this work confirmed that *bla*$_{\text{CTX-M-15}}$ is the most prevalent enzyme in Kuwaiti hospitals and the causative enzyme of cefotaxime resistance. Fatherly, the results of this work agree with previous studies from Kuwait describing CTX-M-15 to be the commonest ESBL present in Kuwait. In addition, this work reports the first description of other CTX-M-1 group enzymes such as *bla*$_{\text{CTX-M-28}}$, *bla*$_{\text{CTX-M-55}}$, and *bla*$_{\text{CTX-M-117}}$ in the Middle East and in Kuwait.

Despite of the wealth of reports from Kuwait reporting the increased prevalence of *bla*$_{\text{CTX-M-15}}$, factors leading to their increased spread and acquisition were not studied in depth. Therefore, this project focused on studying the genetic platforms of *bla*$_{\text{CTX-M-15}}$ and possible genetic elements associated with its spread. GW-PCR, simplex PCR, plasmid sizing and conjugation studies served this purpose. The results of GW-PCR indicated that the commonest element of the upstream arrangement of all of the reported *bla*$_{\text{CTX-M-15}}$ genes and some CTX-M-1 group enzymes was IS*Ecp1*. IS*Ecp1* could possibly be the main factor for the increased spread of *bla*$_{\text{CTX-M-15}}$ and responsible for HGT. The genetic environment studies also proved the variability of the genetic platforms carrying *bla*$_{\text{CTX-M-15}}$. Additionally, all of the reported CTX-M-1 enzymes in this work were found to be transferrable.

To accomplish the last objective of this project, studying the mutational effects was done by structural analysis. In this work, the mutational changes of the reported CTX-M-1 group enzymes with different amino acids were visualized using specialized software. From structural modelling, these amino acid changes could possibly affected the conformation of the active site of the describe enzymes and consequently their hydrolytic profiles towards cephalosporins.

7 References

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8 Appendices

Appendix (A)

ATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTCAC
GCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAA
AACTTGCCGATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTA
ACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATG
TGCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAG
CGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTA
ACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAG
CTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGAT
TGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCCCGACAGCTGGGAG
ACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACACCGCCATTCCG
GGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCGGAA
TCTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACAT
GGATGAAAGGCAATACCACCGGTGCAGCGAGCATTCAGGCTGGACTGCCTGCT
TCCTGGGTTGTGGGGGATAAAACCGGCAGCGGTGGCTATGGCACCACCAACGA
TATCGCGGTGATCTGGCCAAAAGATCGTGCGCCGCTGATTCTGGTCACTTACT
TCACCCAGCCTCAACCTAAGGCAGAAAGCCGTCGCGATGTATTAGCGTCGGCG
GCTAAAATCGTCACCGACGGTTTG**TAA**

Nucleotide sequence of isolates with *bla*$_{\text{CTX-M-15}}$ gene. This sequence is obtained from isolate number 4, 15, 17, 20, 37, 47, 55, 57, 60, 61, 74, 75, 80, 82, 87, 88, 90, 91, 92, 93, 94, 95, 96, 98, 99, 100, 101, and 105. The start codon is highlighted in green colour, while the stop codon is coloured in red. Our isolates share ≥ 98% identity with CTX-M-15 gene (GenBank accession № HQ157357.1).

ATGGCGACGGCAACCGTCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCA
AACGGCGGACGTACAGCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCA
GACTGGGTGTGGCATTGATTAACACAGCAGATAATTCGCAAATACTTTATCGT
GCTGATGAGCGCTTTGCGATGTGCAGCACCAGTAAAGTGATGGCCGCGGCCGC
GGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTTAAATCAGCGAGTTGAGA
TCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAAT
GGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGATAA
CGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCG
CGTTCGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCG
ACGTTAAACACCGCCATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGC
AATGGCGCAAACTCTGCGGAATCTGACGCTGGGTAAAGCATTGGGCGACAGCC
AACGGGCGCAGCTGGTGACATGGATGAAAGGCAATACCACCGGTGCAGCGAGC
ATTCAGGCTGGACTGCCTGCTTCCTGGGTTGTGGGGGATAAAACCGGCAGCGG
TGGCTATGGCACCACCAACGATATCGCGGTGATCTGGCCAAAAGATCGTGCGC
CGCTGATTCTGGTCACTTACTTCACCCAGCCTCAACCTAAGGCAGAAAGCCGT
CGCGATGTATTAGCGTCGGCGGCTAAAATCGTCACCAACGGTTTG**TAA**

Nucleotide sequence of isolates with *bla*$_{\text{CTX-M-28}}$ gene. This sequence is obtained from isolate number 86. The start codon is highlighted in green colour, while the stop codon is coloured in red. Our isolate shares 98% identity with CTX-M-28 gene (GenBank accession № EU531510).

ATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTCAC
GCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAA
AACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATT
AACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGAT
GTGCAGCACCAGTAAAGTGATGGCCGTGGCCGCGGTGCTGAAGAAAAGTGAAA
GCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTT
AACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGA
GCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGA
TTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCCCGACAGCTGGGA
GACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACACCGCCATTCC
GGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCGGA
ATCTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACA
TGGATGAAAGGCAATACCACCGGTGCAGCGAGCATTCAGGCTGGACTGCCTGC
TTCCTGGGTTGTGGGGGATAAAACCGGCAGCGGTGGCTATGGCACCACCAACG
ATATCGCGGTGATCTGGCCAAAAGATCGTGCGCCGCTGATTCTGGTCACTTAC
TTCACCCAGCCTCAACCTAAGGCAGAAAGCCGTCGCGATGTATTAGCGTCGGC
GGCTAAAATCGTCACCGACGGTTTG**TAA**

Nucleotide sequence of isolates with *bla*$_{\text{CTX-M-55}}$ gene. This sequence is obtained from isolate number 89. The start codon is highlighted in green colour, while the stop codon is coloured in red. Our isolate shares 98% identity with CTX-M-55 gene (GenBank accession № HM748991.1).

ATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTCAC
GCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAA
AACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATT
AACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGAT
GTGCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAA
GCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTT
AACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGA
GCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGA
TTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCCCGACAGCTGGGA
GACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACACCGCCATTCA
GGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCGGA
ATCTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACA
TGGATGAAAGGCAATACCACCGGTGCAGCGAGCATTCAGGCTGGACTGCCTGC
TTCCTGGGTTGTGGGGGATAAAACCGGCAGCGGTGGCTATGGCACCACCAACG
ATATCGCGGTGATCTGGCCAAAAGATCGTGCGCCGCTGATTCTGGTCACTTAC
TTCACCCAGCCTCAACCTAAGGCAGAAAGCCGTCGCGATGTATTAGCGTCGGC
GGCTAAAATCGTCACCGACGGTTTG**TAA**

Nucleotide sequence of isolates with *bla*$_{\text{CTX-M-117}}$ gene. This sequence is obtained from isolate number 97. The start codon is highlighted in green colour, while the stop codon is coloured in red. Our isolate shares 98% identity with CTX-M-117 gene (GenBank accession № JN227085).

ATGGTTAAAAAATCACTGCGCCAGTTCACGCTGATGGCGACGGCAACCGTCAC
GCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACAGCAAA
AACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATT
AACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGAT
GTGCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAA
GCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTT
AACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGA
GCTTAGCGCGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGA
TTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTTCGCCCGACAGCTGGGA
GACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACACCGCCATTCC
GGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCGGA
ATCTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACA
TGGATGAAAGGCAATACCACCGGTGCAGCGAGCATTCAGGCTGGACTGCCTGC
TTCCTGGGTTGTGGGGGATAAAACCGGCAGCGGTGACTATGGCACCACCAACG
ATATCGCGGTGATCTGGCCAAAAGATCGTGCGCCGCTGATTCTGGTCACTTAC
TTCACCCAGCCTCAACCTAAGGCAGAAAGCCGTCGCGATGTATTAGCGTCGGC
GGCTAAAATCGTCACCGACGGTTTG**TAA**

Nucleotide sequence of isolates with *bla*$_{\text{CTX-M-3}}$ gene. This sequence is obtained from isolate number 102. The start codon is highlighted in green colour, while the stop codon is coloured in red. Our isolate shares 98% identity with CTX-M-3 gene (GenBank accession № GQ339102.2).

Appendix (B)

Met V K K S L R Q F T L **Met** A T A T V T L L L G S V P
L Y A Q T A D V Q Q K L A E L E R Q S G G R L G V A L
I N T A D N S Q I L Y R A D E R F A **Met** C S T S K
V **Met** A A A A V L K K S E S E P N L L N Q R V E I K K
S D L V N Y N P I A E K H V N G T **Met** S L A E L S A A
A L Q Y S D N V A **Met** N K L I A H V G G P A S V T A F
A R Q L G D E T F R L D R T E P T L N T A I P G D P R
D T T S P R A **Met** A Q T L R N L T L G K A L G D S Q R
A Q L V T W **Met** K G N T T G A A S I Q A G L P A S W V
V G D K T G S G G Y G T T N D I A V I W P K D R A P L
I L V T Y F T Q P Q P K A E S R R D V L A S A A K I V
T D G L **Stop**

Protein sequence of isolates encoding *bla*$_{CTX\text{-}M\text{-}15}$. Met is the start codon ATG; highlighted with green colour. The Stop codon is TAA.

Met A T A T V T L L L G S V P L Y A Q T A D V Q Q K L

A E L E R Q S G G R L G V A L I N T A D N S Q I L Y R

A D E R F A **Met** C S T S K V **Met** A A A A V L K K S E S

E P N L L N Q R V E I K K S D L V N Y N P I A E K H V

N G T **Met** S L A E L S A A A L Q Y S D N V A **Met** N K L

I A H V G G P A S V T A F A R Q L G D E T F R L D R T

E P T L N T A I P G D P R D T T S P R A **Met** A Q T L R

N L T L G K A L G D S Q R A Q L V T W **Met** K G N T T G

A A S I Q A G L P A S W V V G D K T G S G G Y G T T N

D I A V I W P K D R A P L I L V T Y F T Q P Q P K A E

S R R D V L A S A A K I V T N G L **Stop**

Protein sequence of isolates encoding *bla*$_{\text{CTX-M-28}}$. Met is the start codon ATG; highlighted with green colour. The Stop codon is TAA.

Met V K K S L R Q F T L **Met** A T A T V T L L L G S V P

L Y A Q T A D V Q Q K L A E L E R Q S G G R L G V A L

I N T A D N S Q I L Y R A D E R F A **Met** C S T S K

V **Met** A A A A V L K K S E S E P N L L N Q R V E I K K

S D L V N Y N P I A E K H V N G T **Met** S L A E L S A A

A L Q Y S D N V A **Met** N K L I A H V G G P A S V T A F

A R Q L G D E T F R L D R T E P T L N T A I Q G D P R

D T T S P R A **Met** A Q T L R N L T L G K A L G D S Q R

A Q L V T W **Met** K G N T T G A A S I Q A G L P A S W V

V G D K T G S G G Y G T T N D I A V I W P K D R A P L

I L V T Y F T Q P Q P K A E S R R D V L A S A A K I V

T D G L **Stop**

Protein sequence of isolates encoding *bla*$_{CTX-M-55}$. Met is the start codon ATG; highlighted with green colour. The Stop codon is TAA.

Met V K K S L R Q F T L **Met** A T A T V T L L L G S V P

L Y A Q T A D V Q Q K L A E L E R Q S G G R L G V A L

I N T A D N S Q I L Y R A D E R F A **Met** C S T S K

V **Met** A A A A V L K K S E S E P N L L N Q R V E I K K

S D L V N Y N P I A E K H V N G T **Met** S L A E L S A A

A L Q Y S D N V A **Met** N K L I A H V G G P A S V T A F

A R Q L G D E T F R L D R T E P T L N T A I P G D P R

D T T S P R A **Met** A Q T L R N L T L G K A L G D S Q R

A Q L V T W **Met** K G N T T G A A S I Q A G L P A S W V

V G D K T G S G D Y G T T N D I A V I W P K D R A P L

I L V T Y F T Q P Q P K A E S R R D V L A S A A K I V

T D G L **Stop**

Protein sequence of isolates encoding *bla*$_{\text{CTX-M-117}}$. Met is the start codon ATG; highlighted with green colour. The Stop codon is TAA.

Met V K K S L R Q F T L **Met** A T A T V T L L L G S V P

L Y A Q T A D V Q Q K L A E L E R Q S G G R L G V A L

I N T A D N S Q I L Y R A D E R F A **Met** C S T S K

V **Met** A V A A V L K K S E S E P N L L N Q R V E I K K

S D L V N Y N P I A E K H V N G T **Met** S L A E L S A A

A L Q Y S D N V A **Met** N K L I A H V G G P A S V T A F

A R Q L G D E T F R L D R T E P T L N T A I P G D P R

D T T S P R A **Met** A Q T L R N L T L G K A L G D S Q R

A Q L V T W **Met** K G N T T G A A S I Q A G L P A S W V

V G D K T G S G G Y G T T N D I A V I W P K D R A P L

I L V T Y F T Q P Q P K A E S R R D V L A S A A K I V

T D G L **Stop**

Protein sequence of isolates encoding *bla*$_{CTX-M-3}$. Met is the start codon ATG; highlighted with green colour. The Stop codon is TAA

Characterization of IncFIA, IncFIB, and IncN Plasmids Carrying CTX-M-3, -15, -55 β-lactamases from *Escherichia coli* and *Klebsiella pneumoniae* Strains from 5 Major Kuwaiti Hospitals

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Objectives: To characterize plasmids carrying CTX-M-3, -15, -55 in *E. coli* and *K. pneumoniea* strains isolated from 5 major hospitals in Kuwait. **Methods:** Ten strains of *E. coli* and 6 *K. pneumoniae* with ESBL production were collected from 5 major hospitals in Kuwait. Further detection of CTX-M enzyme activity was determined using PCR (Dutour *et al.*, 2002). The minimum inhibitory concentrations (MICs) of the selected strains were determined using four classes of antibiotics; 3 different generations of cephalosporins, fluoroquinolones, aminoglycoside, and carbapenems (Andrews, 2010, Andrews, 2009, Andrews, 2001). The epidemiologic relatedness of the selected strains was determined by pulsed-field gel electrophoresis (PFGE) (Durmaz *et al.*, 2009). The strains were fatherly sequenced and their plasmids were typed with PCR-based replicon typing method (Carattoli *et al.*, 2005). **Results:** With PCR, all of the studying strains were shown to express CTX-M enzyme activity. All of the strains were highly resistant to all antibiotic classes except for carbapenems. Using PFGE, there was no epidemiologic relatedness among the studying strains.

Upon sequencing, CTX-M-15 was found in 14 strains, CTX-M-3 and CTX-M-55 in one strain each. The gene was carried on IncFIA, IncFIB, and IncN plasmids. **Conclusion:** CTX-M-15 was found to be predominant among all of the strains. The strains were shown to express increased resistance to all antibiotic classes, which directs treatment regimens towards carbapenems. The absence of epidemiologic relatedness among the studying strains suggests the variability of the plasmids carrying $bla_{\text{CTX-M-15}}$ gene. This result is supported by the presence of different plasmid types carrying $bla_{\text{CTX-M-15}}$ gene.